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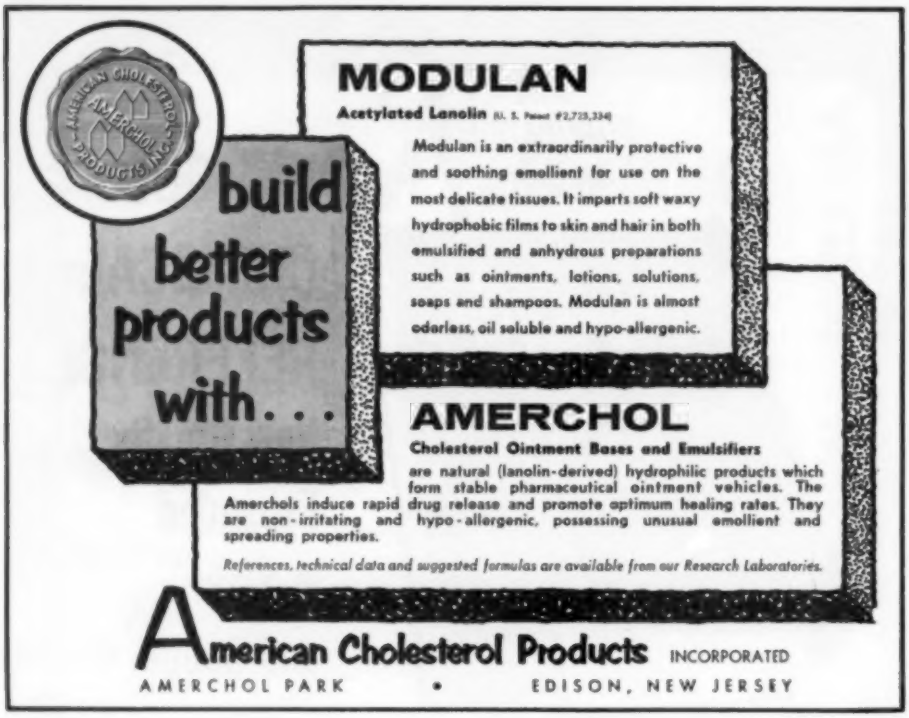
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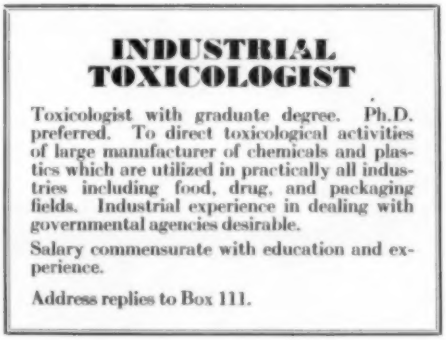
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JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

VOLUME XLVIII NOVEMBER 1959 NUMBER 11

# A Simplified Procedure for the Determination of Vitamin A*

By JOSEPH A. NAPOLI, BERNARD Z. SENKOWSKI, and ALEX. E. MOTCHANE

**A modification of the U. S. P. XV procedure is described. The proposed procedure shortens considerably the time for analysis. It also improves the precision of the unsaponifiable values by at least a factor of two. The precision of the Morton-Stubbs correction factor remains unchanged. The method is shown to be applicable to a wide range of products. A discussion of solvent purification and instrument checking and calibration is included.**

THE WIDELY USED PROCEDURE for the quantitative analysis of vitamin A in various products is described in the U. S. P. XV (1). It represents the experience accumulated in numerous industrial and scientific laboratories over the past ten or fifteen years. The improvement proposed consists of the use of a single extraction in place of the multiple extractions of the U. S. P. XV procedure. As a consequence, a notable increase in the precision is obtained. The U. S. P. XV reports an expected discrepancy in results of different laboratories using the prescribed method of approximately 2 S. D. = ±8 per cent at $P = 0.05$. With this magnitude of error, it has been necessary to carry out four determinations in order to obtain a corrected average value of about ±2 per cent ($P = 0.05$).

Several attempts have been made toward improving the method and its precision (2–4) but none has found its way into general practice. In particular, Mulder's (5) recent work should be mentioned for a thorough analysis of the problem and a novel solution. Our work was done very nearly at the same time.

* Received July 16, 1959, from the Control Spectrophotometric and Physical Chemistry Laboratories of Hoffmann-La Roche Inc., Nutley, N. J.

Our sincere thanks to E. G. E. Shafer, Director of Products Control, for his helpful advice and to Dr. F. Forrester of the Technical Development Department, Analytical Division, for supplying the data in Table III.

The modification of the U. S. P. XV method which we have developed eliminates at least one major source of variability and significantly cuts the time for the analysis. At the same time the precision of the unsaponified values is at least doubled.

## RESULTS

Several series of comparisons are presented between results obtained by the new method and by the U. S. P. XV.

The variations of the individual potency values in Table I are obviously completely out of range from each other and could be treated together only on a relative scale. Such a uniform scale is easily obtained by adjusting all values to the same arbitrarily chosen level, i. e., using appropriate factors to make the averages of individual samples to read, for instance, 1.000 or any other convenient figure. Conventional simple statistics then become meaningful, keeping in mind that although the variations between samples have been suppressed, the number of different groups of samples used reduces the number of the degrees of freedom available for comparison.

This procedure is justified by the technique of measurement, which is based on spectrophotometric readings, and dictated mainly by its limitations. All such readings are in the range of $E$ between 0.5 and 0.8, and are, in practice, even within somewhat closer limits (see footnote, Table V) This is accomplished, as is well known, by adjusting weights and dilutions. The direct readings could not be compared at all without adjustment for weights. The error is related directly to the experimental results which are these very closely similar readings. The differences between the samples are given, on the other hand, mainly by the weights and dilution factors by which all errors are being multiplied at the same time as the readings. The actual variations in the final results must be, therefore, proportional to the dilution factors, i. e., in the end, to the amount of vitamin A. They are obviously very different with different samples, whereas the relative

errors remain the same. These differences in factors are seen to be accidental, and consequently irrelevant, as far as the relative error is concerned.

The precision indexes were obtained by the analysis of variance. The results are collected in Fig. 1 and Table V.

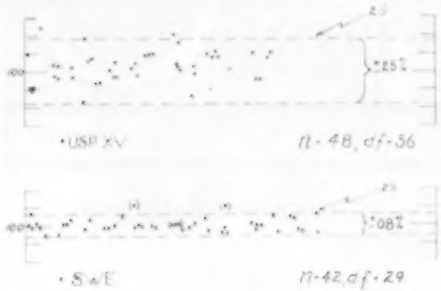


Fig. 1.—Random errors in vitamin A determinations, multiple extraction (U. S. P. XV) *vs.* single (S.W.E.).

## DISCUSSION

In the discussion of our results, we wish to stress two points. The first point is purely technical: a single extraction obviously eliminates one source of error, namely multiple transfers of ether extracts. Such transfers can be made quantitatively, but this may be difficult to achieve with uniform precision in routine operations. This source of error has never been properly evaluated. The number of shakings to which at least some of the vitamin A present is subjected is reduced considerably, and this also may be of importance.

The second point refers to the system of solvents used. The extraction with one large volume of ether (150 ml.) radically changes the composition of the phases. Vitamin A is actually extracted in one operation, the phases being pure water—containing soap, if present—and ether containing 20% alcohol, plus, possibly, very dilute soap. There is no question that this extraction is quantitative, i. e., that the distribution is decisively shifted in favor of the ether layer.

It is not known at present which of these changes is of greater importance. The second is more fundamental, but not necessarily more decisive.

The maximum absorption, as shown in Table IV, was found as high in the new procedure (S. W. E.[1]) as in the U. S. P. XV. The regression coefficient of S. W. E. on U. S. P. XV values was, for the data in Table I, 1.0005 ± 0.002; Table II, 1.006 ± 0.014, the limits of error being 2 S. D., as in Table V. We are investigating the possibility for which there are a few indications that a slightly higher recovery might be possible. The important point, for the time being remains that the recovery by the new procedure is at least as good as by U. S. P. XV.

The precision of the uncorrected values, $E(1\%, 1\text{ cm.})$, is, on the other hand, considerably better than the U. S. P. XV, as seen from Table V. The error was about two to three times smaller and this result highly significant (at the level of $P = 2.5$ to 0.5%), (Fig. 1).

[1] S. W. E. is used to denote the single-water-ether method as proposed in this report.

On the other hand, the precision of the correction factor ($F\ MS = Ac$ corr./$A$ unc.) was not improved (see Tables). The number of data seems to be sufficient to consider this difference as real. At present, we have no definite explanation for this. It appears that we have obtained a separation of the effect on the reading at the maximum, and of the effect on readings on the slopes of the curve: the former became considerably less variable, whereas the latter, or at least their combined effect, remained as variable as before. We abstain from discussing several possibilities which, for the time being, must be speculative only. As a practical conclusion, the precision of the analytical results will be improved with respect to U. S. P. XV when the correction factor is greater than 0.97, since then the correction is omitted: in short, when dealing with material of high spectral purity. The studies now in progress may show the way to improve precision in all cases.

The last point we wish to mention, ratio $S/W$, represents an additional proof of the soundness of the new procedure. The height of the maximum of the whole sample ($W$) before, and the height ($S$) after saponification are important physical data which are used much too little. Provided the absorption measured are due to vitamin A only, the absorption after saponification must be higher than before, since the molecular absorption of the vitamin A alcohol is higher than of the esters. The following comparison (Table VI) is based on data from Table I.

The independent significance of this index is in the possibility afforded for checking the analysis when the whole value is available, as is often the case, without reference to another sample or standard. The measurement of a direct dilution of the whole sample is generally easy to make with relatively high precision and accuracy. A ratio near 1.04 confirms the validity of the reading after saponification, and conversely, and perhaps, more critically, a significantly lower ratio is a sure indication of an error, loss, or a discrepancy of some kind. A value slightly higher than 1.04 is the ratio of the molecular absorption of the free vitamin A alcohol and of the acetate, which can be derived, for instance, from data published by Cama, *et al.* (6), as early as 1951. In our experience it holds for the palmitate as well as the acetate, and it was found helpful in our laboratories, as indicated, at least since that time.

## CONCLUSIONS

Replacement of the four ether extractions called for in the U.S.P. XV procedure for vitamin A determination by one extraction with a fivefold volume of ether results in a partial, but significant, improvement in operation and results. Notably, two things are achieved: the time of analysis is reduced to almost one-half, and the precision of the absorption measured is increased two to three times. The full benefit of these improvements is felt in cases where the measured values represent the final result, i. e., with high purity vitamin A, diluted or undiluted, showing a U. S. P. purity factor $f\ MS$ of more than 0.97, and therefore not requiring correction (1).

On the other hand, the precision of the correction factor was not improved. In this way, apparently,

a separation has appeared for the first time in experimental data of variations affecting the maximum of the absorption curve, as distinct from the slopes. This matter shall be discussed later on the basis of new experiments. Considerable improvement seems possible in this direction.

## PROCEDURE

**Sampling.**—A sample of not less than 100 mg. is recommended in order to have a precision of 0.1% or better depending on the balance used. Also, the weighed sample should contain not less than 10,000 units of vitamin A.

High potency material, crystalline or liquid, as such or diluted in oil, is best weighed directly into a volumetric flask (25-ml.), dissolved by adding 1 ml. of ethyl ether, and bringing to mark with isopropyl alcohol. A 5-ml. aliquot is transferred to the saponification flask. Powders are sampled in a weighing bottle, transferred to a saponification flask, and 7 ml. glycerin added. Multivitamin liquid preparations are weighed directly into the saponification flask. In preparations containing vitamin

TABLE I.—U. S. P. XV VS. SINGLE EXTRACTION PROCEDURE (S. W. E.)

| Sample (u./Gm) × $10^6$ | $E$ (1%, 1 cm.) U. S. P. XV Unsaponifiable Fraction | $f$ (MS)[a] | $S/W$[b] | $E$ (1%, 1 cm.) S. W. E. Unsaponifiable Fraction | $F$ (MS)[a] | $S/W$[b] | Direct Whole u./Gm |
|---|---|---|---|---|---|---|---|
| 2.9 | 1571 | 1.0148 | ... | 1559 | 0.9739 | ... | 1495 |
| | 1571 | 1.0150 | ... | 1558 | 0.9575 | ... | ... |
| | Av. 1571 | 1.0149 | 1.050 | 1561 | 0.9827 | ... | ... |
| | ... | ... | ... | 1558 | 0.9705 | ... | ... |
| | ... | ... | ... | Av. 1559 | 0.9712 | 1.043 | ... |
| 1.8 | 931.5 | 0.9630 | ... | 936.9 | 0.9998 | ... | 906.5 |
| | 919.2 | 0.9602 | ... | 945.6 | 1.0066 | ... | ... |
| Sample 1 | 931.8 | 0.9596 | ... | 945.6 | 1.0113 | ... | ... |
| | 923.9 | 0.9459 | ... | 946.8 | 1.0045 | ... | ... |
| | Av. 926.9 | 0.9572 | 1.022 | Av. 943.7 | 1.0056 | 1.041 | ... |
| 1.8 | 964.1 | 1.0000 | ... | 960.0 | 0.9909 | ... | 919.0 |
| | 958.2 | 0.9950 | ... | 966.0 | 0.9950 | ... | 917.0 |
| Sample 2 | 982.3 | 0.9527 | ... | Av. 963.0 | 0.9930 | 1.049 | Av. 918.0 |
| | 940.7 | 1.0263 | ... | ... | ... | ... | ... |
| | Av. 961.4 | 0.9950 | 1.047 | ... | ... | ... | ... |
| 1.0 | 618.1 | 0.9324 | ... | 625.7 | 0.9630 | ... | 602.1 |
| Sample 1 | 618.7 | 0.9275 | ... | 625.7 | 0.9581 | ... | ... |
| | 609.0 | 0.9350 | ... | 626.3 | 0.9650 | ... | ... |
| | 609.3 | 0.9343 | ... | 630.0 | 0.9689 | ... | ... |
| | Av. 613.8 | 0.9301 | 1.019 | Av. 626.9 | 0.9640 | 1.041 | ... |
| 1.0 | 615.4 | 0.9221 | ... | 620.9 | 0.9643 | ... | 585.8 |
| | 618.9 | 0.9534 | ... | 619.9 | 0.9725 | ... | 589.1 |
| Sample 2 | 612.8 | 0.9677 | ... | Av. 620.4 | 0.9684 | 1.056 | Av. 587.5 |
| | 612.8 | 0.9625 | ... | ... | ... | ... | ... |
| | Av. 615.0 | 0.9464 | 1.067 | ... | ... | ... | ... |
| 0.5 | 323.8 | 0.9916 | ... | 323.4 | 0.9905 | ... | ... |
| (Dry pellets) | 325.3 | 1.0038 | ... | 323.4 | 0.9940 | ... | ... |
| | 326.3 | 0.9739 | ... | 321.7 | 1.0048 | ... | ... |
| | 324.3 | 0.9718 | ... | 323.6 | 1.0069 | ... | ... |
| | Av. 324.9 | 0.9853 | ... | Av. 323.0 | 0.9992 | ... | ... |
| | ... | ... | ... | 320.0 | 0.9820 | ... | ... |
| | ... | ... | ... | 320.8 | 1.0011 | | |
| | ... | ... | ... | Av. 320.4 | 0.9916 | ... | ... |
| 0.325 | 202.6 | 0.9793 | ... | 197.5 | 0.9984 | ... | ... |
| (Dry pellets) | 193.4 | 0.9582 | ... | 195.9 | 1.0167 | ... | ... |
| Sample 1 | 202.8 | 0.9643 | ... | Av. 196.7 | 1.0076 | ... | ... |
| | 202.7 | 0.9718 | ... | ... | ... | ... | ... |
| | Av. 200.4 | 0.9684 | ... | ... | ... | ... | ... |
| 0.325 | 216.5 | 1.0038 | ... | 208.2 | 0.9670 | ... | ... |
| (Dry pellets) | 210.4 | 0.9909 | ... | 208.9 | 0.9677 | ... | ... |
| Sample 2 | 214.1 | 0.9691 | ... | 208.1 | 0.9848 | ... | ... |
| | 197.4 | 0.9827 | ... | 211.8 | 0.9452 | ... | ... |
| | Av. 209.6 | 0.9866 | ... | Av. 209.3 | 0.9662 | ... | ... |
| 0.10 | 53.03 | 1.011 | ... | 54.01 | 1.067 | ... | 51.99 |
| U. S. P. Vitamin A | 51.85 | 1.047 | ... | 53.85 | 1.059 | ... | 51.07 |
| Reference | 53.64 | 1.054 | ... | 53.94 | 1.058 | ... | 50.75 |
| Soln. (I) | 52.75 | 1.050 | ... | 53.92 | 1.059 | ... | 51.85 |
| | 51.22 | 1.013 | ... | Av. 53.93 | 1.056 | 1.053 | 50.85 |
| | 53.52 | 1.058 | ... | ... | ... | ... | 50.66 |
| | 53.72 | 1.067 | ... | ... | ... | ... | 51.95 |
| | 52.10 | 1.053 | ... | ... | ... | ... | 51.10 |
| | 53.55 | 1.055 | ... | ... | ... | ... | 50.88 |
| | Av. 52.82 | 1.045 | 1.031 | ... | ... | ... | Av. 51.23 |

TABLE I. (*Continued*)

| Sample (u./Gm.) × 10³ | *E* (1%, 1 cm.) U. S. P. XV Unsaponifiable Fraction | *f* (MS)[a] | *S/W*[b] | *E* (1%, 1 cm.) S. W. E. Unsaponifiable Fraction | *F* (MS)[a] | *S/W*[b] | Direct Whole u./Gm. |
|---|---|---|---|---|---|---|---|
| 0.04 | 33.71 | 0.7462 | ... | 33.73 | 0.7830 | ... | ... |
| Vitamin A | 33.87 | 0.7599 | ... | 33.41 | 0.7783 | ... | ... |
| "Natural" from fish oils | Av. 33.79 | 0.7531 | 1.027 | Av. 33.57 | 0.7807 | 1.020 | 32.9 |
| 0.001 | 5.933 | 0.9098 | ... | 5.827 | 0.9166 | ... | ... |
| Vitamin A in | 5.827 | 0.8873 | ... | 5.818 | 0.9132 | ... | ... |
| aqueous | 5.828 | 0.9398 | ... | 5.789 | 0.8996 | ... | ... |
| multi- | Av. 5.863 | 0.9118 | ... | 5.769 | 0.9187 | ... | ... |
| vitamin | ... | ... | ... | Av. 5.801 | 0.9120 | ... | ... |
| prepn. | | | | | | | |
| 0.06 | 37.51 | 0.9282 | ... | 38.70 | 0.9105 | ... | ... |
| Vitamin A | 37.50 | 0.8996 | ... | 39.22 | 0.8982 | ... | ... |
| with | 38.25 | 0.9207 | ... | Av. 38.95 | 0.9044 | ... | ... |
| β-carotene | 38.38 | 0.9125 | ... | ... | ... | ... | ... |
| in oil | Av. 37.91 | 0.9153 | ... | ... | ... | ... | ... |

[a] A corr./A uncorr. (1). $F\,(MS) = A_{325\,corr.} = \dfrac{6.815\,(A_{325} - 2.5554\,(A_{310}) - 2.60\,(A_{334})}{A_{325}}$.

[b] $S/W = \dfrac{A_{325}\text{ (after saponification)}}{A_{325}\text{ (direct measurement, no saponification)}}$, i. e. unsap. fraction over direct whole

Data in this table refer to samples of widely different potencies. (48 values from 12 samples as analyzed by two chemists in Laboratories I and II.)

A and β-carotene in oil, the carotene is removed by weighing about 1 Gm.[2] into an amber, glass-stoppered centrifuge tube, adding 20 ml. of isopropyl alcohol, gassing with nitrogen, shaking on a shaking machine for ten minutes, and centrifuging. A 5-ml. aliquot of the clear supernatant solvent is placed in the saponification flask.

**Saponification.**—To the sample in the 125-ml. saponification flask is added sufficient 95% U. S. P. alcohol to make a total volume of about 30 ml., followed by 3 ml. of 50% w/w potassium hydroxide.

Displace the air by nitrogen, reflux on a steam bath for thirty minutes, and cool rapidly under the tap to room temperature.

**Extraction.**—With all samples except dry vitamin A powders, add 30 ml. of water into the saponification flask and transfer quantitatively into an amber 250-ml. separatory funnel (Squibb type[3]).

In the case of dry vitamin A powders, add only 20 ml. water before transferring, then rinse the saponification flask with the remaining 10 ml. of water in order to pick up any deposit which may have formed during saponification. Now add 2 Gm. hydrated sodium sulfate, finely powdered.

Rinse the saponification flask with three 50-ml. portions of ethyl ether, collecting them in the separator. (Rinse the top of the flask while it is inverted over the separatory funnel to prevent run back.)

Displace air with nitrogen, stopper, shake vigorously for about two minutes, and let stand till the layers separate completely; five minutes usually suffices. Add a few drops of water if the boundary is not clearly visible.

**Washing.**—Discard the aqueous layer leaving one drop above the stopcock. Add 60 ml. distilled water, swirl gently, and wait till the layers separate completely. Discard the water, add another 50 ml. of water, displace the air with nitrogen, close, and gently tumble the funnel. Repeat this treatment two more times, shaking a little more vigorously. Discard the water layers.

**Dilution.**—Transfer the entire ethyl ether extract to a 250-ml. amber volumetric flask, rinsing the separator several times with small portions of ethyl ether and collecting them in the flask, and bring to mark with ether. Subdilute with isopropyl alcohol. The final concentration of ether in the measured solution should not exceed 5%. This solution should contain between 10 and 15 units of vitamin per ml.

**Measurement.**—Measure the absorbance at the following wavelengths four times each, using a suitable spectrophotometer: 310 mμ, 325 mμ, 334 mμ. Average the values for each wavelength and proceed with the calculation.

Calculate using the following expression:

$$E(1\%, 1\text{ cm.}), (325\ m\mu) = \frac{E \times V}{W \times 100} \times \frac{1}{L}$$

where $E$ = average absorbance at 325 mμ, $V$ = calculated final volume containing $W$ (grams) of sample, in ml., $W$ = weight of sample in grams, and $L$ = path length in cm.

Correction for irrelevent absorption: calculate according to U. S. P. XV (1).

$$A_{(325\ corr.)} = 6.815\,(A_{325}) - 2.555\,(A_{310}) - 4.260\,(A_{334})$$

In the case of oils containing β-carotene the volume of isopropyl alcohol added to dissolve the vitamin A and separate it from the β-carotene is increased by the oil present. This volume change must be corrected for as follows: $Vc = 20 + \Delta v$, $\Delta V$ = weight of sample in grams × 1.16, $Vc$ = volume corrected, 1.16 = the increase of the volume of 20 ml. of isopropyl alcohol per 1 Gm. of oil containing β-carotene and vitamin A as obtained from the experimentally determined straight line which passed through the origin.

[2] This weight should not exceed 1.2 Gm.

[3] No grease is employed; the stopcocks (4 mm. bore) are lubricated with water only.

TABLE II.—U. S. P. XV vs. S. W. E. DRY VITAMIN A PELLETS[a]

| Sample | U. S. P. XV E (1%, 1 cm.) | U. S. P. XV F (MS)[b] | S. W. E. E (1%, 1 cm.) | S. W. E. F (MS)[b] | Sample | U. S. P. XV E (1%, 1 cm.) | U. S. P. XV F (MS)[b] | S. W. E. E 1%, 1 cm.) | S. W. E. F (MS)[b] |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 204.7 | 0.9849 | 204.8 | 0.9688 | 12 | 206.3 | 0.9364 | 209.7 | 0.9521 |
| | 206.0 | 1.0045 | 210.4 | 0.9982 | | 203.0 | 0.9228 | 210.7 | 0.9166 |
| | Av. 205.4 | 0.9947 | 207.6 | 0.9835 | | Av. 204.7 | 0.9296 | 210.2 | 0.9344 |
| 2 | 200.8 | 0.9415 | 199.3 | 0.9408 | 13 | 210.5 | 0.9159 | 212.1 | 0.9589 |
| | 199.2 | 0.9135 | 200.1 | 0.9492 | | 212.8 | 0.9228 | 212.7 | 0.9377 |
| | Av. 200.0 | 0.9275 | 199.7 | 0.9450 | | Av. 211.7 | 0.9194 | 212.4 | 0.9483 |
| 3 | 195.6 | 1.0031 | 199.1 | 0.9877 | 14 | 215.4 | 0.9596 | 222.9 | 0.9486 |
| | 192.8 | 1.0003 | 199.6 | 0.9800 | | 211.6 | 0.9534 | 223.7 | 0.9228 |
| | Av. 194.2 | 1.0017 | 199.4 | 0.9839 | | Av. 213.5 | 0.9517 | 223.3 | 0.9357 |
| 4 | 205.6 | 1.0129 | 204.5 | 0.9828 | 15 | 211.2 | 0.9950 | 215.9 | 0.9670 |
| | 203.3 | 1.0171 | 205.4 | 0.9786 | | 211.1 | 0.9534 | 204.8 | 0.9589 |
| | Av. 204.5 | 1.0150 | 205.0 | 0.9807 | | Av. 211.2 | 0.9742 | 210.4 | 0.9630 |
| 5 | 216.1 | 0.9702 | 214.6 | 0.9541 | 16 | 209.2 | 0.9493 | 209.9 | 0.9875 |
| | 212.9 | 0.9847 | 210.0 | 0.9611 | | 210.5 | 0.9561 | 203.5 | 1.0148 |
| | Av. 214.9 | 0.9772 | 212.3 | 0.9576 | | Av. 209.9 | 0.9527 | 206.7 | 1.0012 |
| 6 | 215.7 | 0.9415 | 214.9 | 0.9205 | 17 | 211.2 | 1.0058 | 202.9 | 0.9889 |
| | 215.0 | 0.9534 | 216.2 | 0.9492 | | 212.8 | 1.0079 | 205.7 | 0.9834 |
| | Av. 215.4 | 0.9475 | 215.6 | 0.9349 | | Av. 212.0 | 1.0069 | 204.3 | 0.9862 |
| 7 | 213.7 | 0.9902 | 215.7 | 0.9807 | 18 | 205.7 | 0.9282 | 215.4 | 1.0040 |
| | 216.1 | 0.9882 | 205.4 | 1.0213 | | 207.1 | 0.9405 | 215.7 | 0.9507 |
| | Av. 214.9 | 0.9892 | 210.6 | 1.0010 | | Av. 206.4 | 0.9344 | 215.6 | 0.9774 |
| 8 | 210.2 | 1.0003 | 210.6 | 0.9723 | 19 | 218.1 | 0.9759 | 221.1 | 0.9759 |
| | 206.8 | 0.9660 | 212.6 | 0.9807 | | 201.3 | 0.9317 | 223.7 | 0.9602 |
| | Av. 208.5 | 0.9832 | 211.6 | 0.9765 | | Av. 209.7 | 0.9538 | 222.4 | 0.9680 |
| 9 | 216.5 | 1.0038 | 213.1 | 0.9691 | 20 | 222.6 | 0.9923 | 222.8 | 1.0180 |
| | 214.1 | 0.9909 | 199.3 | 0.9827 | | 222.0 | 1.0093 | 211.2 | 0.9609 |
| | Av. 215.3 | 0.9974 | 206.2 | 0.9759 | | Av. 222.3 | 1.0008 | 217.0 | 0.9894 |
| 10 | 202.7 | 0.9575 | 213.4 | 0.9664 | 21 | 210.4 | 0.9820 | 223.8 | 0.9596 |
| | 208.2 | 0.9357 | 212.4 | 0.9030 | | 211.0 | 1.0209 | 224.4 | 0.9848 |
| | Av. 205.5 | 0.9466 | 212.9 | 0.9347 | | Av. 210.7 | 1.0015 | 224.1 | 0.9722 |
| 11 | 209.9 | 0.9486 | 213.3 | 0.9309 | 22 | 208.2 | 1.0395 | 199.2 | 0.9912 |
| | 209.6 | 0.9337 | 215.8 | 0.9323 | | 208.7 | 1.0269 | 200.6 | 1.0206 |
| | Av. 209.8 | 0.9412 | 214.6 | 0.9316 | | Av. 208.5 | 1.0332 | 199.9 | 1.0059 |

[a] Duplicate measurements on 22 lots of vitamin A pellets as analyzed in Laboratory No. I. [b] See footnote[a] Table I.

## EXPERIMENTAL

**Apparatus.**—Of the Beckman DU spectrophotometers used, one was equipped with a photomultiplier detector and another was equipped with a conventional photocell. In order to obtain greater source stability, our hydrogen lamp power supply units are fed from a 1 KVA Sola constant voltage transformer.

The importance of wavelength calibration cannot be overemphasized and particular care is taken in checking the calibration of our instruments and adjusting the scale. A mercury discharge lamp (supplied by the instrument manufacturer) is used to check periodically our wavelength settings.

The following is a typical control run.

| Known Mercury Lines, mμ | Mercury Lines, Found D265[a] | 506 | 40735 | 113907[a] |
|---|---|---|---|---|
| 253.65 | 253.79 | 253.70 | 253.74 | 253.68 |
| 289.36 | 289.49 | 289.50 | 289.45 | 289.25 |
| 312.57 | 312.67 | 312.62 | 312.77 | 312.54 |
| 313.15 | 313.33 | 313.29 | 313.23 | 313.15 |
| 334.15 | 334.05 | 334.13 | 334.13 | 334.14 |

[a] Instruments used in this study.

Any notable deviation from the expected wavelength is corrected for by following the directions supplied by the manufacturer.

TABLE III.—COMPARISON OF RESULTS BY SINGLE EXTRACTION (S. W. E.) AND U. S. P. XV METHOD

High purity Vitamin A $\left(0.97 \leq \frac{A \text{ corr.}}{A} \leq 1.03\right)$

$\frac{\text{S. W. E.}}{\text{U. S. P. XV}}$ (average of 141 samples) = 1.001

Standard Deviation = ±4%, the magnitude of which is determined by the larger error of the U. S. P. method.

**Instrument Settings.**—Inaccurate instrument settings can be disastrous in this analysis and special care must be exercised in setting the wavelengths accurately. The slit settings are of great importance and care must be taken with this setting, too. It is for that reason, as well as slight source instability, that it is recommended that each reading be taken four times and the average of the four readings used in the subsequent calculations. Any value which deviates more than 2% from the other values is omitted and further checks are made to substantiate the value.

A typical slit program is as follows: 310 mμ, 0.22 mm.; 325 mμ, 0.20 mm.; 334 mμ, 0.18 mm.

**Calibration of Density Readings.**—A solution containing 20.220 mg. of Bureau of Standards potassium dichromate, dried at 140° to constant weight, in 1,000 ml. of 0.05 *N* potassium hydroxide was prepared and

TABLE IV.—SUMMARY OF THE COMPARISONS OF THE SINGLE EXTRACTION PROCEDURE (S. W. E.) AND THE U. S. P. XV

| Table No. | Potency Range $10^2$ u./Gm. | $E$ (1%, 1 cm.) Ratio S. W. E./U. S. P. XV[a] |
|---|---|---|
| I | | |
| Liquid preparations, 46 values | 0.01 to 2.9 | (9)[b] 1.005 (12) 1.000 |
| II | | |
| Vitamin A pellets, 44 values | 0.38 | (22) 1.007 |
| III | | |
| High potency vitamin A, 141 values | 1.3 to 2.9 | (141) 1.000 |

[a] Figures in parentheses are the number of samples.
[b] Liquid preparations

TABLE V.—PRECISION (2 S. D.) OF U. S. P. XV AND OF S. W. E. (SINGLE EXTRACTION) REFERRED TO SAMPLE AVERAGE = 100

| Samples | Degrees of Freedom | Precision of | 2 S. D., % U. S. P. XV | S. W. E. | Variance Ratio | F Level |
|---|---|---|---|---|---|---|
| 12 (Tab. 1[a]) | 36 | $E$(1%, 1 cm.) | 2.5 | 0.8 | 10.6 | 2.6 (0.005) |
| | | F (MS) | 3.0 | 2.5 | 1.2 | ... |
| 22 (Tab. 2) | 22 | $E$(1%, 1 cm.) | 5.7 | 3.7 | 2.4 | 2.36 (0.025) |
| | | $F$(MS) | 3.0 | 4.0 | 0.75 | ... |

| [a] Sample | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| $E$(1%, 1 cm.) | 1,560 | 945 | 960 | 625 | 625 | 325 | 200 | 200 | 54 | 33 | 555 | 39 |
| Readings ($E$ 1 cm.), approx. | | | | | | | | | | | | |
| U. S. P. | 0.65 | 0.70 | 0.60/0.76 | 0.60/0.67 | 0.56/0.70 | 0.69 | 0.62/0.79 | 0.70 | 0.53 | 0.65 | 0.69 | 0.57/0.74 |
| S. W. E. | 0.65 | 0.67 | 0.50/0.55 | 0.56 | 0.58 | 0.68 | 0.54 | 0.52/0.67 | 0.71 | 0.70 | 0.68 | 0.78 |

F(MS), Morton Stubbs correction factor, based on U. S. P. XV (1) calculated directly from ratios = $A$ (corr.)/$A$ (325).

TABLE VI.—RATIO $S/W$, UNCORRECTED (UNSAP. TO WHOLE)

| Sample | 1 | 2 | 3 | 4 | 5 | 9 | 10 | Av. |
|---|---|---|---|---|---|---|---|---|
| U. S. P. XV | 1.050 | 1.022 | 1.047 | 1.019 | 1.047 | 1.031 | 1.027 | 1.030 |
| S. W. E. | 1.043 | 1.041 | 1.049 | 1.041 | 1.056 | 1.053 | 1.020 | 1.044 |

The difference between the averages (S. D. = 0.0124) is not significant at $P$ = 0.05. The ratio is in both cases about 1.04.

measured at 372.5–373 mμ *vs.* 0.05 *N* potassium hydroxide.

| Instrument | $E$ (1%/1 cm.) |
|---|---|
| D265 | 249.4 |
| 506 | 249.3 |
| 40735 | 249.4 |
| 113907 | 249.3 |

These solutions were sealed in 5-cc. ampuls and monthly checks are performed on all instruments using these solutions. A graphical record is kept and any abnormal deviations are immediately investigated. The values shown above agree quite well with the values published by Cama, Collins, and Morton (2). Morton's Beckman, $A$ = 248.2; average of all instruments (in Morton's paper) $A$ = 247.4.

Stability of the instruments is also enhanced by the use of large capacity, slow discharge rate, six-volt batteries.

**Quartz Cells.**—(Fused Quartz cells are recommended.) The quartz cells employed were matched for absorbance by comparing them, after suitable cleaning, using the same solvent in each.

Cleaning is performed by thorough rinsing with the solvent used (isopropanol in the case of water-immiscible solvents) followed in succession by distilled water, soaking in a mild detergent for about one-half hour and adequate distilled water rinsing. The cells are then checked, one *versus* the other. If detergent cleaning does not suffice, immerse them in 30% fuming sulfuric acid for about twenty minutes.[4] This is followed by thorough rinsing with distilled water and checking.

The path length of the cells should be known to at least ±0.5%. The stated value of the cells was checked by two independent methods.

First a cell was measured using standard chromate at the maximum (373 mμ). This value was then confirmed with greater precision by half-filling the cell with mercury and measuring, using a microscope micrometer, the distance between the mercury-quartz boundaries at opposite faces.

**Solvents.**—(*a*) The ethyl alcohol used is 95% U. S. P. ethanol, aldehyde-free. (*b*) The ethyl ether used is Mallinckrodt (black label) special peroxide-free grade (AR) analytical reagent. Daily checks for peroxides are performed with KI solution; however, a more sensitive check is to keep a solution of vitamin A in the ether overnight, or longer, in the refrigerator. This solution should be stable for several days in pure peroxide-free ethyl ether. (*c*) Shell isopropyl alcohol, or one of comparable spectral purity, is passed through a Norite column containing five pounds of Norite for five gallons of isopropanol at the rate of one gallon per day. This

[4] Fused cells only.

is followed by a simple distillation through a 1-meter glass helix column at the rate of one gallon per day. This treatment produces a solvent of adequate spectral purity.

**Glassware.**—All glassware used, except for the pipets, is amber, light-resistant. Careful cleaning is essential. The cleaning procedure recommended is as follows: Rinsing with tap water; soaking in a Calgonite bath overnight; rinsing with tap water; shaking each piece with concentrated chromic acid solution; and rinsing with tap water, followed by distilled water until all the acid is rinsed out. (At least 5 rinsings of each.)

**Balance.**—Periodic checks on the balance and weights are performed by using standardized Bureau of Standards weights.

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# Ethylene Oxide Sterilization of Spores in Hygroscopic Environments*

By JOHN B. OPFELL, JOHN P. HOHMANN, and ARTHUR B. LATHAM

***Bacillus globigii*** **spores were exposed to ethylene oxide after being dried on several types of surfaces. The viability tests, after exposure, showed significant effects were produced by the presence of the hygroscopic substances, glycerin, and filter paper. Absence of hygroscopic substances appeared to increase the resistance of *B. globigii* spores to gaseous ethylene oxide sterilization.**

WATER VAPOR is a factor in ethylene oxide sterilization. Some writers (1, 10, 20) contend that moisture improves the effectiveness whereas others (6–9, 12, 13) have observed the contrary. Methods (5, 11, 14, 20) of inoculating objects to be sterilized have differed among the several investigations. The effect of the inoculation method on the evaluation of the sterilization process was studied. The results of this study suggest a possible explanation for the contradictory observations.

For more than twenty years ethylene oxide has been used to sterilize temperature-sensitive medical equipment. Several investigations of processing conditions have been made. Grundy and co-workers (11) found ethylene oxide sterilized inoculums of 20,000 spores on filter paper in two hours at 26.7°. The concentration of ethylene oxide used was 1,250 mg. per liter. The immediate environment of the spore contained lactose as well as filter paper. Lloyd and Thompson (14) reported that two hours at 55° in 1,000 mg. per liter of ethylene oxide were sufficient to sterilize $10^6$ *B. globigii*, $5 \times 10^5$ *Cl. sporogenes*, and $10^5$ *B. stearothermophilus* spores on filter paper strips when the relative humidity of the ethylene oxide was between 30 and 50 per cent. Eisman (5) showed that ethylene oxide does not completely penetrate large masses of clothing. This is perhaps not too surprising when one considers the mass of absorbed water in clothing which has not been desiccated. At 30°, water as a liquid can absorb as much as 0.4 Gm. (15) of ethylene oxide per Gm. of water. Hall (6–9) claims that spices are easier to sterilize when they are dry. The extent of drying necessary was not specified. The experiment to be described was motivated by observing that washed *B. globigii* spores in large inoculums desiccated on polystyrene could not be inactivated by a process which sterilized these spores dried on filter paper.

The standards for evaluating sterilization are described in the current edition of the "United States Pharmacopeia" (19). Meaningful sterility tests must conform to these standards. In addition, sterility tests are meaningful only when the test organism is specified and is used under defined conditions. As the test organism, most investigations (5, 11, 14, 17, 20) have used spores of *Bacillus subtilis* var. *niger* strain *B. globigii* (ATCC 9372) (3). This organism was used in the experiment to be described. This spore is not necessarily the one most resistant (5) to ethylene oxide exposure under all conditions.

The number of cells in the inoculum and the method of applying the inoculum to the test object are important factors in evaluating a sterilization process. The appropriate number of cells which should be used depends on the number which might occur naturally on the object. The resistance of an inoculum measured by the period of exposure required to render the object sterile,

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The authors wish to acknowledge the help of Dr. Thomas W. Green.

depends on the size of the inoculum. For example, assume that the probability that a spore selected at random from the inoculum would survive an exposure of $t$ minutes under specified processing conditions is:

$$p = e^{-kt} \qquad \text{(Eq. 1)}$$

(The constant $k$ has units of reciprocal minutes.) One can show that the probability of failing to sterilize the object after an exposure of $t$ minutes under the same processing conditions is approximately

$$P = Ne^{-kt} \qquad \text{(Eq. 2)}$$

for large exposure times. The number of spores in the inoculum is $N$. To reduce the probability of failing to sterilize to a very low level, either the size of the inoculum must be reduced or the exposure time must be sufficiently increased. Equations 1 and 2 have been amply confirmed (2, 4, 20) for both ethylene oxide and steam sterilization. In practice, $N$ cannot be reduced below the maximum microorganism population which might naturally inhabit the object. Because microorganisms have finite volumes, $N$ cannot be infinitely large. Usually $N$ is chosen (11, 14) sufficiently large to include an ample safety factor over the natural population size observed to exist in representative samples of similar objects.

Some experimenters (5, 20) have used suspensions of spores deposited and dried on the surface to be sterilized. Others have used small pieces of paper (11) or fabric (20) which have been inoculated with spore suspensions and dried before insertion into containers to be sterilized. Still others have dried suspensions of spores in starch paste or physiological saline, lactose, or glycerine solution on the surface to be inoculated.

Variations in method of inoculating surfaces to be used in proving sterilization have been significant. The effect of such variations was measured in the experiment to be described. To the extent possible, the size of the inoculum was uniform and the ethylene oxide concentration was varied in a systematic manner.

## METHODS

The inside surfaces of tubes one-fourth inch in internal diameter and seven-eighths inch in length were inoculated with *B. globigii* spores. Except for category B, described in Table I, the spores were dried from suspension in distilled water. The spores were washed in distilled water before the final suspension was prepared to free them of hygroscopic and other substances which are not part of their innate structure. In category B the suspension contained 2% glycerin U. S. P. In category C the spores were deposited on the filter paper only. The tubes were inoculated with uniform volumes of the stock spore suspension. The number of viable spores recovered by a single rinse was determined by a plate-counting technique using a representative inoculated tube after it had dried for twenty-four hours in a 32° incubator. While the recovery of spores in categories B, C, and D was roughly the same, relatively fewer were recovered when dried on polystyrene from suspension in distilled water.

For each concentration of ethylene oxide shown in Table I the indicated number of tubes representing each inoculation method were placed together in a 200-ml. glass flask immersed in an air bath thermostat. After evacuation with an oil-sealed mechanical pump, the indicated amount of dry pure ethylene oxide vapor was admitted to the flask. The concentration was computed from the measured pressure in the flask at 37°, the temperature of the air bath after the system had reached equilibrium. Equilibrium was attained in a matter of a very few minutes. The ethylene oxide was left in contact with the inoculated tubes for seventeen hours at 37 ± 1° before it was removed by evacuation and replaced with air filtered through a sterile cotton filter.

Each of the treated tubes was tested for sterility using procedures described in the "United States Pharmacopeia" (19). Each was placed in a test tube containing 40 ml. of fluid thioglycollate medium and incubated there for seven days at 32°. In every experiment two untreated but inoculated controls from category D were combined with two polystyrene tubes, which had not been inoculated, in ten ml. of the medium. The two polystyrene tubes were exposed to the ethylene oxide with the treated tubes. In each case the control test demonstrated the presence of viable *B. globigii* spores. These tests were performed to show that the amount of ethylene oxide absorbed by each of the tubes was insufficient to suppress *B. globigii* proliferation in the medium.

## RESULTS

Table I shows the ratio of the number of tubes which showed no growth of *B. globigii* in the fluid thioglycollate medium to the total number treated. The number of viable spores recovered from a representative sample tube prior to treatment is shown next to the test result.

The effect of the two-fold variation in ethylene oxide concentration was negligible. What effect there was indicated the higher concentrations to be more efficacious. No attempt was made to control the humidity in the glass flask other than to use pure ethylene oxide vapor and to use standardized amounts of the hygroscopic materials.

Because of the identical treatment for each of the four categories, the fractions of success in sterilization may be compared among the categories. Confidence intervals (99%), based on the binomial distribution for each of the four categories, were calculated for the fraction of the tubes sterilized. In Table I these confidence intervals (16) indicate that the differences observed in the effect of ethylene oxide on *B. globigii* spores in cases B and C were significantly greater than those in cases A and D. Such differences could hardly be ascribed to chance.

One might speculate on the cause for such great differences. Both glycerin and paper absorb large quantities of water vapor in comparison with polystyrene and clean glass. Even in the environ-

TABLE I.—EFFECT OF INOCULATION METHOD ON EVALUATION

| Treatment | | Category A—Polystyrene-Distilled Water[a] | | Category B—Polystyrene-2% Glycerin[b] | | Category C—Filter Paper-Distilled Water[c] | | Category D—Glass-Distilled Water[d] | |
|---|---|---|---|---|---|---|---|---|---|
| Absolute Pressure, in. of Mercury | Concentration of Ethylene Oxide, mg./liter | Sterile/Treated | Spores Recovered 1,000's | Sterile/Treated | Spores Recovered 1,000's | Sterile/Treated | Spores Recovered, 1,000's | Sterile/Treated | Spores Recovered, 1,000's |
| 17.24 | 996 | 5/10 | 400 | 10/10 | 400 | 7/10 | 600 | 5/10 | 100 |
| 15.90 | 920 | 1/10 | 200 | 8/10 | 700 | 10/10 | 60 | 1/10 | 900 |
| 15.41 | 892 | 9/9 | 200 | 10/10 | 300 | 10/10 | 400 | 10/10 | 800 |
| 15.25 | 883 | 5/5 | 3 | 5/5 | 40 | 5/5 | 40 | 3/4 | 10 |
| 15.14 | 876 | 5/5 | 100 | 5/5 | 300 | 5/5 | 500 | 0/5 | 400 |
| 15.00 | 868 | 1/5 | 40 | 5/5 | 400 | 5/5 | 400 | 2/5 | 200 |
| 14.43 | 835 | 8/10 | 100 | 10/10 | 600 | 10/10 | 600 | 6/10 | 500 |
| 14.08 | 815 | 3/10 | 50 | 10/10 | 500 | 10/10 | 300 | 3/10 | 300 |
| 13.59 | 787 | 4/10 | 50 | 10/10 | 300 | 9/10 | 500 | 7/10 | 400 |
| 13.48 | 780 | 2/10 | 100 | 10/10 | 200 | 5/10 | 300 | 1/10 | 400 |
| 13.07 | 756 | 3/10 | 400 | 10/10 | 400 | 7/10 | 500 | 5/10 | 400 |
| 12.96 | 750 | 3/10 | 500 | 10/10 | 700 | 6/10 | 700 | 6/10 | 700 |
| 12.96 | 750 | 4/10 | 1000 | 10/10 | 3000 | 6/10 | 2000 | 2/10 | 2000 |
| 12.95 | 749 | 0/10 | 500 | 9/10 | 700 | 10/10 | 700 | 5/10 | 800 |
| 12.61 | 730 | 5/10 | 40 | 10/10 | 500 | 10/10 | 400 | 2/10 | 500 |
| 12.37 | 716 | 0/10 | 300 | 0/10 | 300 | 0/10 | 400 | 0/10 | 500 |
| 11.25 | 650 | 4/10 | 300 | 10/10 | 400 | 9/10 | 400 | 9/10 | 400 |
| 9.09 | 525 | 6/10 | 100 | 10/10 | 600 | 9/10 | 400 | 10/10 | 100 |
| Totals | | 66/164 | ... | 152/165 | ... | 131/165 | ... | 77/164 | ... |
| Confidence interval (99%) | | 0.30 to 0.50 | | 0.85 to 0.97 | | 0.72 to 0.88 | | 0.37 to 0.57 | |

[a] Distilled water suspension of spores was dried on the inside of polystyrene tubes.
[b] Two per cent glycerin suspension of spores was dried on the inside of polystyrene tubes.
[c] Distilled water suspension of spores was dried on filter paper which was inside polystyrene tubes.
[d] Distilled water suspension of spores was dried on the inside of glass tubes.

ment of the 32° incubator, glycerin and paper can absorb appreciable moisture (18) when the ambient relative humidity is 30% or higher. The slow release of this moisture into the ethylene oxide which is initially free of water vapor will maintain high relative humidity in the vicinity of the spore, for at least a portion of the exposure to ethylene oxide. The amount of paper used in each test (10 strips each weighing 10 mg.) was insufficient to affect greatly the humidity of the ethylene oxide (15) remote from the paper. In a sterilizer containing a large quantity of cellulosic materials, the humidity of the ethylene oxide may be quite high after equilibrium is reached. This is particularly true if these materials were in equilibrium with high humidity air before entering the sterilizer. For sterilization, the amount of water vapor which must be added to the gas appears to depend on the amount of water absorbed by the material supporting the bacterial spore. Too much absorbed moisture may strip the ethylene oxide from the gas. Too little moisture in the gas may spare the microorganisms.

Variations in the relative humidity in the vicinity of the spores appear to have a greater effect on the sterilization than do variations in the concentration of ethylene oxide. These factors might have significance with respect to the sterilization process as well as to methods of evaluation.

## SUMMARY

Glycerin and/or filter paper in immediate contact with dried *B. globigii* spores reduced their resistance to ethylene oxide exposure significantly. In designing sterilization processes for nonhygroscopic materials and in designing evaluation methods for sterilization of any material, these results indicate how important truly representative inoculation techniques may be.

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# Esters of Erythromycin IV*

## Alkyl Sulfate Salts

By V. C. STEPHENS, J. W. CONINE, and H. W. MURPHY

**The alkyl sulfate salts of various erythromycin[1] esters were prepared by esterification of erythromycin followed by a double decomposition salt formation. These compounds, and in particular propionyl erythromycin lauryl sulfate, are virtually tasteless and are unaffected by mixing with gastric acid. These properties render them very useful in the preparation of flavored suspensions.**

MANY SALTS of erythromycin have been extensively evaluated and a number of them are used commercially. The esters of erythromycin, while somewhat weaker bases than the parent antibiotic, form stable salts with relatively strong acids, as was previously reported (1). The long chain fatty acids, such as oleic and stearic acid, which have been employed to modify the taste of erythromycin, do not form stable salts with erythromycin esters.

In the last paper of this series (2) it was shown that propionyl[2] erythromycin[3], when ingested by fasting patients, produces antibiotic levels in the blood which are greatly superior to those produced by erythromycin itself. The utilization of this more effective compound in other forms of oral medication besides capsules is of great importance. In particular, a flavored suspension could be used advantageously in many patients who find it difficult or impossible to swallow a capsule.

The formulation of propionyl erythromycin into such a flavored suspension has been complicated by two of its characteristics: (*a*) Although dry propionyl erythromycin has only a faintly bitter taste, its flavor in an aqueous suspension is very unpleasant; and (*b*) The exposure of propionyl erythromycin, like erythromycin itself, to gastric juice results in a rapid loss in antibiotic activity.

In view of this acid instability of propionyl erythromycin, one can explain its high degree of effectiveness in capsule form by assuming that while the capsule will normally disintegrate within the stomach, the contents are largely protected by a very low wettability until they have passed into the intestine. Lower antibiotic blood levels are obtained when propionyl erythromycin is given with food than when it is administered orally to fasting patients. When a wetting agent is added to the contents of the capsule a markedly lower level is also produced[4] (Fig. 1). A flavored suspension would be completely wet before ingestion and would thus provide a maximum opportunity for contact with the gastric contents.

We have prepared and evaluated a number of salts of propionyl erythromycin and other erythromycin esters. Of the various salt types tested, the alkyl sulfate salts, such as propionyl erythromycin lauryl sulfate[5] (PELS), have been found to be very useful, especially in the preparation of flavored suspensions.

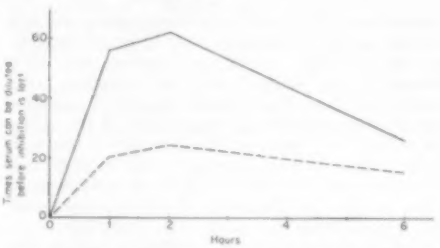


Fig. 1.—Crossover blood levels of 10 fasting subjects, 250-mg. dose in capsules. Solid line, propionyl erythromycin; broken line, propionyl erythromycin plus 5% polyoxyethylene sorbitan monooleate.

The chemical structure of PELS is given in Fig. 2. Table I contains a brief summary of the alkyl sulfate salts which were prepared and evaluated. Since they are similar in properties, only PELS will be discussed in detail. PELS is easily prepared from erythromycin by esterification followed by a double decomposition reaction with sodium lauryl sulfate to form the salt. The method of preparation is outlined in the experimental section of this report. All of the other salts listed in Table I were prepared by the same general method. The melting points are not given since all of the compounds melt, with decomposition, at about the same point. The exact point of decomposition is somewhat de-

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[1] Ilotycin is the Lilly trade name for erythromycin.

The authors wish to express their appreciation to the various members of Eli Lilly and Co. who have aided and cooperated in obtaining the data reported.

[2] While an "ate" ending is commonly employed for both esters and salts, in this paper the ester group will be named as a prefix and the salt as a suffix, i. e., propionyl erythromycin acetate rather than erythromycin propionate acetate.

[3] Ilosone is the Lilly trade name for propionyl erythromycin ester.

[4] The blood levels reported were obtained from Dr. R. S. Griffith of the Lilly Clinical Research Laboratories.

[5] Ilosone Lauryl Sulfate is the Lilly trade name for propionyl erythromycin ester lauryl sulfate.

TABLE I.—PHYSICAL CHARACTERISTICS OF ALKYL SULFATES

| X | Y | Assays Erythromycin Theory,[a] mcg./mg | Chem. | Biol. | $SO_4$, % Theory[a] | Found |
|---|---|---|---|---|---|---|
| Acetyl | Lauryl | 680 | 664 | 675 | 8.9 | 8.8 |
| Propionyl | Lauryl | 674 | 650 | 639 | 8.8 | 8.7 |
| Acrylyl | Lauryl | 675 | 623 | 680 | 8.8 | 8.7 |
| Crotonyl | Lauryl | 669 | 593 | 416 | 8.7 | 8.9 |
| *n*-Butyryl | Lauryl | 666 | 597 | 635 | 8.7 | 8.8 |
| Methoxyacetyl | Lauryl | 665 | 637 | 673 | 8.6 | 8.4 |
| *n*-Valeryl | Lauryl | 657 | 598 | 635 | 8.6 | 8.5 |
| Iso-Valeryl | Lauryl | 657 | 602 | 562 | 8.6 | 8.2 |
| Ethyl carbonyl | Lauryl | 665 | 664 | 608 | 8.7 | 8.5 |
| Ethyl succinyl | Lauryl | 630 | 554 | 548 | 8.2 | 8.7 |
| Propionyl | Octyl | 714 | 676 | 680 | 9.3 | 9.7 |
| Propionyl | 2-Ethyl hexyl | 714 | 721 | 722 | 9.3 | 9.0 |
| Propionyl | Tetradecyl[b] | 657 | 636 | 646 | 8.6 | 8.5 |
| Propionyl | Heptadecyl[c] | 630 | 562 | 572 | 8.2 | 8.5 |
| Propionyl | Stearyl | 624 | 613 | 670 | 8.1 | 8.2 |
| Chloroacetyl | Lauryl | 661 | 648 | ... | 3.19[d] | 3.04 |
| α-Chloropropionyl | Lauryl | 655 | 605 | ... | 3.12[d] | 2.70 |

[a] Theoretical values are corrected for about 3% moisture content.
[b] 2-Methyl-7-ethyl undecyl-4-sulfate.
[c] 3,9-Diethyl tridecyl-6-sulfate.
[d] Chlorine analyses. Theoretical content corrected for moisture.

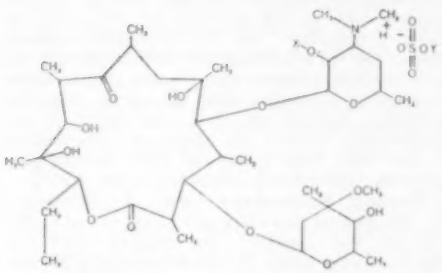

Fig. 2—Structure of acyl erythromycin alkyl sulfates. For PELS, X is $CH_3CH_2C(=O)-$; and Y is $C_{12}H_{25}$.

pendent on the rate of heating. PELS melts, with decomposition, between 135–140°.

PELS labeled with $S^{35}$ was prepared as outlined in the experimental section in about 90 per cent yield.

The water solubility of PELS is extremely low. By the usual physiochemical measurements, only an approximate water solubility could be obtained. By assaying a saturated aqueous solution for erythromycin content by the ultraviolet method commonly employed (3), the water solubility was found to be about 0.024 mg. per cc. This is approximately one-twelfth the solubility of propionyl erythromycin and one-fortieth that of erythromycin. With this very low solubility it is not surprising that the compound is substantially tasteless. PELS can be readily formulated into a pleasantly flavored aqueous suspension.

As was previously mentioned, when propionyl erythromycin and erythromycin are mixed with pooled human gastric juice they dissolve and their antibiotic activity is quickly lost. Salts of carboxylic acids, even though they are water insoluble, behave similarly. The strong acid of gastric juice liberates the free carboxylic acid and dissolves the antibiotic as the soluble hydrochloride salt. PELS, however, is a salt of a very strong acid (lauryl sulfuric acid). The gastric acid is not strong enough to displace the acid radical of the salt and, when mixed with gastric juice, it remains undissolved and retains its potency even when exposed to the acid for extended periods (Table II). The blood levels produced

TABLE II.—GASTRIC JUICE STABILITIES

| Compound | Exposure Time, min. | Activity Retained at 37°, % |
|---|---|---|
| Erythromycin[a] | 5 | 3.5 |
| Ethyl carbonyl erythromycin[a] | 5 | 5.7 |
| Erythromycin stearate[a] | 5 | 2 |
| Propionyl erythromycin[b] | 5 | 5.4 |
| Propionyl erythromycin saccharinate[b] | 15 | 10 |
| Propionyl erythromycin lauryl sulfate[b] | 40 | 97.3 |

[a] pH 1.28. [b] pH 1.10.

by PELS are not significantly different in the fasting and nonfasting subject (Fig. 3). If anything, the peak level is attained somewhat sooner when the antibiotic is ingested with food. This may be due to a greater dispersion of the drug particles or to an increased production of digestive fluids.

Recent work reported by Schanker, *et. al* (4, 5), on the mechanism of drug absorption has shown that organic compounds are probably absorbed in the unionized form. It was stated in

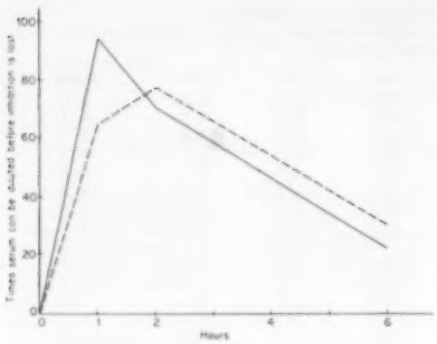


Fig. 3—Crossover blood levels for 10 subjects, 250-mg. dose of a flavored suspension of PELS. Solid line, with food; broken line, without food.

the first of these reports (4) that basic drugs having pKa's of less than 8 were readily absorbed while stronger bases were absorbed very slowly. If this proposed mechanism is valid for erythromycin and its derivatives, the greater absorption of the propionyl ester (pK′a 6.9) as compared to erythromycin (pK′a 8.6) may be readily explained.

The use of a salt such as PELS would not be expected to affect this absorption process markedly. In a buffered system, such as the intestinal contents, the ratio of ionized to unionized material in solution would be dependent on the pH rather than on the form administered. Since both propionyl erythromycin and PELS probably pass into the intestine largely in the solid state, their relative solubilities in water might exert some influence on the rates of absorption. Our very limited experience with an aqueous suspension of propionyl erythromycin and our previous work with ethyl carbonyl erythromycin have indicated that both of these compounds are more quickly absorbed than PELS, but the latter appears to give a somewhat longer effective level.

Blood samples from subjects who received propionyl erythromycin, as the base or as PELS, were extracted with chloroform and the antibiotic factors separated by paper chromatography. In every case the blood contained both erythromycin and its propionyl ester. No significant difference appeared as a result of the form of the drug administered. The ratio of esterified to free erythromycin varies from subject to subject and according to the interval between ingestion and blood withdrawal.

The very poor blood levels obtained with erythromycin esters in which the acid radical contains five or more carbons (2) are not readily explained by this mechanism.

## EXPERIMENTAL

**Propionyl Erythromycin Lauryl Sulfate (PELS) from Propionyl Erythromycin.**—Propionyl erythromycin (32.6 Gm.) was dissolved in 300 cc. of anhydrous methanol-free acetone. After filtration, a filtered solution of purified sodium lauryl sulfate (12.8 Gm.) in 200 cc. of distilled water was added. A solution of 4.4 cc. of glacial acetic acid in 20 cc. of water was then introduced, followed by another 175 cc. of water which was added rapidly while stirring. The desired salt formed very quickly and was removed by filtration. It was washed several times with water and dried in a vacuum oven at 50°. The yield of long white needles was 41.5 Gm.

**PELS from Erythromycin.**—Propionic anhydride (85 cc.) was added to a solution of 395 Gm. of erythromycin dissolved in 1,500 cc. of anhydrous methanol-free acetone. It was stirred briefly, filtered, and allowed to stand for two hours at room temperature. By the procedure outlined above, the ester solution was utilized directly to form the lauryl sulfate salt. In this case no additional acid is needed since the excess propionic anhydride and the propionic acid formed in the esterification convert the propionyl erythromycin to the ionic form (propionyl erythromycin propionate). The yield was 555 Gm. or about 95%.

**Propionyl Erythromycin Lauryl $S^{35}$ Sulfate[6].**—By the general procedure given, 430 mg. of erythromycin was esterified and reacted with 145 mg. of sodium lauryl $S^{35}$ sulfate containing 2.5 mc. of $S^{35}$. The yield was 460 mg. of PELS having a specific activity of 6 μc. per mg.

**Stability Study in Pooled Human Gastric Juice.**—Pooled normal gastric juice (50 cc.) was warmed to 37–39° in a constant temperature water bath and 100 mg. of propionyl erythromycin (or other compound) was added. The mixture was stirred constantly. Samples for analysis (1 cc. each) were removed at different time intervals and were quickly transferred to 10-cc. volumetric flasks containing 30 mg. of sodium bicarbonate in 2 cc. of water. It was mixed immediately. To each flask 4 cc. of methanol was added, and then sufficient distilled water to give 10 cc. This solution was allowed to stand eighteen hours at room temperature then assayed microbiologically for erythromycin activity.

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[6] Prepared in cooperation with Robert E. McMahon, Organic Chemical Division, The Lilly Research Laboratories.

# Pharmacology and Toxicology of Propionyl Erythromycin Ester Lauryl Sulfate*

By R. C. ANDERSON, C. C. LEE, H. M. WORTH, and P. N. HARRIS

**Clinical usage of erythromycin[1] and propionyl erythromycin ester[2] has confirmed the low toxicity in experimental animals as reported from these laboratories. Propionyl erythromycin ester lauryl sulfate[3] (PELS) has been found to have very low water solubility and is easily usable in pleasantly flavored suspensions. Chronic toxicity studies in rats and dogs have demonstrated the absence of visceral or hematopoietic damage following large doses for over three months. Oral administration of PELS produced higher serum concentrations in rats than propionyl erythromycin. Both antibiotics are mainly absorbed from the intestine of rats and excreted in very small quantities in the bile. High concentrations of erythromycin activity were found in the lung, spleen, liver, kidney, and heart after oral administration. The results of absorption studies using $S^{35}$ labeled PELS will be discussed.**

CLINICAL EXPERIENCE has confirmed the efficacy and low toxicity of erythromycin since it was discovered in 1952 by McGuire, *et al.* (1), of these laboratories. Various salts and esters have been used in different pharmaceutical forms. The crystalline base has been used in ointments and tablets coated with cellulose acetate phthalate (CAP). Erythromycin ethyl carbonate has been employed in drops or suspensions for pediatric therapy. Erythromycin glucoheptonate has been prescribed for intravenous injection. Extensive research revealed that certain esters, when administered orally in capsules, gave earlier, higher, and more consistent blood levels than were obtained with the base or with CAP-coated tablets. Since our publication of the pharmacology and toxicology of propionyl erythromycin (2), Stephens, *et al.* (3), found that certain unique properties of the lauryl sulfate salt of propionyl erythromycin make it possible to prepare a tasteless suspension which produces high and prolonged blood levels. The pharmacological and toxicological studies of this antibiotic are summarized in this report.

## MATERIALS AND METHODS

Propionyl erythromycin ester lauryl sulfate (PELS) assayed 650 mcg. of erythromycin activity/mg. Propionyl erythromycin assayed 880 mcg. of erythromycin activity/mg. These antibiotics were given by weight or by erythromycin activity as stated in different sections of this report.

**Acute Toxicity.**—PELS, suspended in a 5% solution of gum acacia, was given orally to 40 albino mice and 10 albino rats and subcutaneously to 10 albino rats. All animals were fasted overnight and observed for seven days. Six mongrel dogs were given single doses in capsules and observed for any side effects.

**Chronic Toxicity.**—*Rats.*—PELS was administered to rats by the drug-diet method described by Anderson, *et al.* (4). A total of 60 albino rats, weighing 80 to 100 Gm., was used. Five groups of 12 each, equally divided as to sex, were fed diets containing 0, 0.05, 0.1, 0.25, and 0.5% of PELS, respectively. These concentrations were equivalent to 0, 0.031, 0.062, 0.155, 0.31% of erythromycin. Food and water were available *ad libitum* and the daily food intake was recorded.

*Dogs.*—Eight pure-bred beagle hounds, weighing between 8.3 and 13.7 Kg., were given PELS orally in capsules at dose levels of 50–100 mg./Kg. (31–62 mg. of erythromycin activity/Kg.) in two divided doses daily including Saturdays and Sundays. The first dose was administered at 8:00 a.m., the second six to seven hours later. From the twenty-second day a pharmaceutical formulation was used in capsules each of which contained 250 mg. of erythromycin activity. Two to four capsules (36–137 mg. of erythromycin activity/Kg., equivalent to 58–221 mg. of PELS by weight/Kg.) were given daily in divided doses. Careful observations were made for side effects. Blood and urine specimens were collected periodically for various analyses. Blood concentration of the antibiotic was determined at various intervals following the morning dosage. After seventy days of treatment, four dogs were sacrificed and examined grossly. Tissues from the heart, lungs, liver, spleen, kidneys, gastrointestinal tract, and thymus, thyroid, pancreas, and adrenal glands were fixed and stained for microscopic studies. The remaining four dogs continue on test.

**Pharmacodynamics.**—Three mongrel dogs were anesthetized with sodium phenobarbital, 150 mg./Kg. by vein. The carotid artery was cannulated and blood pressure recorded with a mercury manometer. A cannula was also placed in the trachea and respiration recorded with a tambour through a Haley respirometer. A balloon was placed in the duodenum to record intestinal movements. Electrocardiograms were from the standard Lead II and recorded with a Grass polygraph. PELS, as a 10% suspension in 5% acacia, was injected through a catheter opening into the intestine distal to the balloon.

* Received August 21, 1959, from The Lilly Research Laboratories, Eli Lilly and Co. Indianapolis 6, Ind.

The authors wish to thank members of the Biochemical, Physicochemical, and Pharmacological Divisions of the Lilly Research Laboratories for their assistance during various phases of these studies.

Presented to the Scientific Section, A. PH. A., Cincinnati meeting, August, 1959.

[1] Ilotycin is the Lilly trade name for erythromycin.

[2] Ilosone is the Lilly trade name for propionyl erythromycin.

[3] Ilosone Lauryl Sulfate is the Lilly trade name for propionyl erythromycin ester lauryl sulfate.

**Absorption, Distribution, and Excretion.**—*Rats.*—One hundred and fifty male albino rats, weighing 125 to 150 Gm., were fasted overnight. A single dose of 25 mg./Kg. of propionyl erythromycin based on the antibiotic activity was administered orally to 75 rats in the form of a 1% water suspension. The remaining 75 rats received an equivalent dose of PELS. Groups of 15 rats each were decapitated at one-half, one, two, four, and six hours and the serum from each rat was assayed for antibiotic activity.

Twenty-four fasted albino rats, weighing 200 to 225 Gm., were anesthetized with 150 mg./Kg. of phenobarbital sodium intraperitoneally. A double ligature was placed at the junction between the stomach and the duodenum through a medial incision. The bile duct of each rat was cannulated with polyethylene tubing, which was brought out through the incision. A single dose of 100 mg./Kg. of PELS, as a 2% water suspension, was introduced into the stomach of 12 rats with a 22-gauge needle. The remaining 12 rats received the same dose intraduodenally. The incision was closed with wound clips and the bile was collected in graduated centrifuge tubes. At the end of two hours all rats were decapitated. Antibiotic activity was assayed on the serum and the bile appropriately diluted with saline.

The bile duct of 16 fasted albino rats, weighing 225 to 250 Gm., was cannulated with polyethylene tubing under ether anesthesia. The tubing was brought out through the right posterior abdominal wall and the incision was sutured. Ten of these rats were given orally a single dose of 100 mg./Kg. of PELS and six rats received the same dose of propionyl erythromycin. They were kept in Bollman's restrictive cages (5). The bile was collected in graduated centrifuge tubes at intervals and assayed for erythromycin activity. Food and physiological saline were supplied *ad libitum*.

To study blood partition of this antibiotic, 20 female albino rats, weighing 200 to 225 Gm., were fasted overnight. A single dose of 100 mg./Kg. of PELS as a 2% water suspension was given orally. At the end of one hour and four hours 10 rats each were anesthetized with ether and blood was drawn from the abdominal aorta and heparinized. Erythromycin assays were made on the hemolyzed whole blood, on the hemolyzed saline-washed cells, and on the plasma. The hematocrit was also determined.

Tissue distribution was studied in 40 female albino rats, weighing 200 to 225 Gm. They were fasted overnight. A single dose of 100 mg./Kg. of PELS as 2% water suspension was given orally to 20 rats and the same amount of propionyl erythromycin to the remaining 20 rats. Groups of 10 rats each were decapitated at the end of two hours and seven hours after administration. Blood samples were collected separately and the serum was assayed for antibiotic activity. The spleen, kidneys, brain, and a sample of about 0.5 Gm. of the liver and 0.5 Gm. of the lung were removed immediately and homogenized in a glass homogenizer with three volumes of 0.2 *M* phosphate buffer (pH 7.2). The heart was cut into small pieces and homogenized with five volumes of the buffer. All tissue homogenates were well shaken before assay for erythromycin activity. Assays of tissue homogenates from control rats receiving no drug revealed no erythromycin activity. When known quantities of PELS or propionyl erythromycin were added to the various tissues, the recovery of antibiotic activity varied from 64–84%. No correction was made to compute the tissue concentration of erythromycin from treated rats.

*Dogs.*—Four female mongrel dogs with duodenal fistulas, weighing between 12.2 and 15.7 Kg., were fasted for twenty-four hours. A single dose of 25 mg./Kg. of PELS in capsule was administered orally or intraduodenally through the fistula, followed by 30 ml./Kg. of water through a stomach tube. At intervals blood samples were drawn from the jugular vein. Assays were made on the serum and on the urine appropriately diluted with saline.

**Absorption of PELS-$S^{35}$ and Sodium Lauryl Sulfate-$S^{35}$.**—The PELS-$S^{35}$ was prepared by Stephens, *et al.*, of these laboratories (3). Each mg. of PELS-$S^{35}$ contained 6 $\mu$c. of radioactivity. About 10 $\mu$c. of this material plus 16 mg. of "cold" PELS were administered orally to eight albino rats, weighing between 180 to 220 Gm., as a suspension in 2 ml. of water. Four rats were killed by ether inhalation at one hour and the remaining four at four hours. All blood was withdrawn from each animal by heart puncture. The entire gastrointestinal tract, from the esophagus through the rectum, the liver, and kidneys, was removed. All tissues and the blood were digested in warm 1 *N* KOH in ethanol, and counted in Diotol (6) with a tri-carb liquid scintillation counter (Packard Instrument Co., LaGrange, Ill., Model 314).

Another group of eight rats was given 2 ml. of a sodium lauryl sulfate-$S^{35}$ solution which contained 4.4 $\mu$c./ml. The specific activity was 81.5 $\mu$c./mg. The gastrointestinal tract, the liver, the kidneys, and the blood were removed, digested, and examined in the same manner as the tissues of the previous group of rats treated with radioactive antibiotic.

**Microbiological Assay.**—Erythromycin activity was determined by an adaptation of the Food and Drug Administration *Sarcina lutea* cup-plate assay (7) in undiluted serum, in urine and bile appropriately diluted with physiological saline, in hemolyzed whole blood and in hemolyzed washed red blood cells, in plasma, and in various tissue homogenates. All findings were compared against a standard sample of erythromycin base. Horse serum, hemolyzed whole blood and hemolyzed washed red blood cells of rats, rat plasma, and 0.2 *M* phosphate (pH 7.2) were used for setting up the standard curves for the appropriate determinations. It was found that horse serum, dog serum, and rat serum gave the same standard curves.

## RESULTS

**Acute Toxicity.**—The $LD_{50}$'s of PELS in mice after oral and in rats after oral and subcutaneous administration were found to be greater than 6.45 Gm./Kg. During the observation period all animals appeared to be completely normal. Previous work from these laboratories reported the $LD_{50}$ of propionyl erythromycin by oral route to be 2.87 Gm./Kg. for the mouse and >5 Gm./Kg. for the rat (2). Thus, PELS is less toxic to the

mouse and no more toxic to the rat than propionyl erythromycin. The results in dogs are shown in Table I. The incidence of vomiting was much less frequent than has been reported previously with propionyl erythromycin (2).

TABLE I.—TOLERATED DOSES OF PELS IN DOGS

| Dog Number | 8:30 a. m. Dose mg./Kg. | Effects Before 1:30 p. m. | 1:40 p. m. Dose mg./Kg. | Effects Before 4:15 p. m. |
|---|---|---|---|---|
| 3,362 | 25 | No action | 200 | No action |
| 1,775 | 25 | No action | 200 | 3:00 p. m. Vomited |
| 262 | 50 | No action | 50 | No action |
| 4,524 | 50 | No action | 50 | 2:23 p. m. Vomited |
| 4,514 | 100 | No action | 100 | No action |
| 4,526 | 100 | No action | 100 | No action |

**Chronic Toxicity.**—*Rats.*—The mean growth curves for the female rats fed various concentrations in the diet are found in Fig. 1 and for males in Fig. 2. Growth on the 0.05% diet was similar to the control, whereas rats fed 0.1 to 0.5% of PELS did not grow so rapidly as those fed control diets. The average daily food intake was somewhat less in groups showing a lower growth curve. After eighty-one days, half of the rats in each group were sacrificed and submitted to necropsy. Gross and microscopic examination of the heart, lungs, liver, spleen, kidneys, gastrointestinal tract, thymus, thyroid, pancreas, adrenals, salivary glands, and mesenteric lymph nodes revealed no apparent abnormalities. Terminal blood counts were within normal ranges. Terminal blood levels of this antibiotic showed higher values in rats fed higher concentrations. The other 30 rats remain on test.

*Dog.*—The body weight of all dogs remained relatively constant throughout the experiment. No apparent side effect was observed in any dog. In the four sacrificed animals, bone marrow, erythrocyte, leukocyte and differential counts, and hematocrit and hemoglobin determinations remained within normal ranges. Whole blood clotting time and clot retraction time did not change in any dog. Blood sugar and nonprotein nitrogen values showed the usual variations. No glycosuria was noted. Albuminuria was seen so rarely and was of such low degree as to be considered within normal expectation in this species (8).

Serum levels of this antibiotic were determined on these dogs at seventeen, fifty-one, and sixty-five days. All eight dogs showed significant concentrations following administration of the drug. Approximately eighteen hours after the last dose, the serum contained no detectable amount of this antibiotic.

**Pharmacodynamics.**—Three dogs received 36 and 72 mg./Kg. of PELS (equivalent to 25 and 50 mg./Kg. of propionyl erythromycin). In two of the animals the blood pressure began to fall one and one-half to two hours after 36 mg./Kg. to 78 to 81% of the normal rate and remained at this level for more than three hours. The third dog showed no significant change. Following 72 mg./Kg., one dog had no change in blood pressure, one showed an increase of 18%, and the third a fall of 22%.

In two of the three dogs the heart rate was increased by 10–15 beats per minute following 36 mg./Kg. The third dog showed no change. The increase in heart rate followed the fall in blood pressure and was probably compensatory in nature. All three animals had an increase in heart rate following 72 mg./Kg. This varied from 10–15 beats per minute. No significant changes were noted in the configuration of the electrocardiogram after either dose.

None of the animals showed significant changes in the respiratory rate following either 36 or 72 mg./Kg. Intestinal motility was stimulated in two dogs after 36 mg./Kg. and in all dogs after 72 mg./Kg.

**Absorption, Distribution, and Excretion.**—*Serum Levels.*—The serum levels in rats receiving 25 mg./Kg. of erythromycin propionate or of PELS orally are shown in Fig. 3. The serum levels of antibiotic activity produced by PELS were much higher than those obtained with propionyl erythromycin. A peak of serum concentration in rats receiving PELS was reached after two hours, while the propionyl erythromycin produced a maximal serum concentration after one hour. At the end of six hours following administration there were significant amounts of antibiotic in the serum of rats receiving PELS. On the other hand, only a trace of anti-

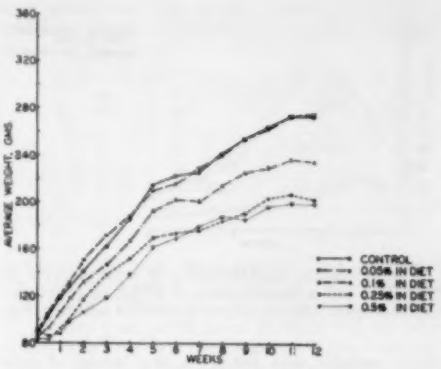


Fig. 1.—The growth curves of female rats fed diets containing PELS.

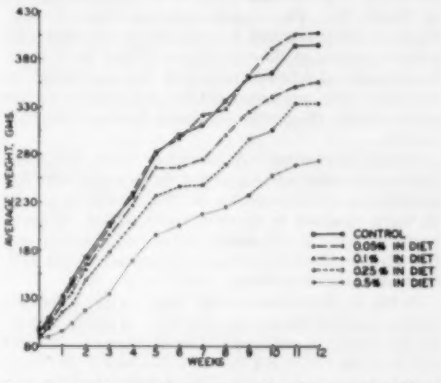


Fig. 2.—The growth curves of male rats fed diets containing PELS.

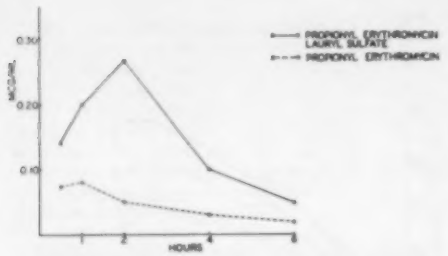


Fig. 3.—Serum concentration of erythromycin in rats after oral administration of PELS or propionyl erythromycin, 25 mg. of erythromycin activity/Kg.

biotic activity was found in the serum of rats receiving the propionyl erythromycin.

The average serum concentrations of erythromycin in four dogs with duodenal fistula are summarized in Table II. The serum concentration of antibiotic activity reached a peak two hours after oral administration of 25 mg./Kg. of PELS in capsule. Intraduodenal administration of the same does to the same dogs produced earlier and slightly better serum levels than were obtained by oral administration.

*Site of Absorption.*—As shown in Table III, when the rat's stomach was ligated at the junction with the duodenum, administration of PELS directly to the stomach resulted in no detectable amount of antibiotic activity in the serum at the end of two hours. Intraduodenal administration produced serum concentrations in all animals.

*Urinary Excretion.*—Four dogs with duodenal fistula excreted an average of 0.61% of orally administered PELS in six hours. An average of 0.59% of the dose was excreted in the urine when this antibiotic was given to the duodenum through the fistula.

*Biliary Excretion.*—Results in Table III show that only 0.07% of the administered dose was excreted in the bile of rats in two hours when PELS was introduced into the duodenum that was ligated at the junction with the stomach. The bile concentration of erythromycin averaged 2.1 mcg./ml. These rats excreted an average of 0.02% of the antibiotic when it was administered into the stomach, although no detectable amount of the antibiotic was found in the serum at the end of two hours.

As shown in Table IV, an average of 1.54% of the orally administered dose of PELS was recovered in the bile of rats after twenty-four hours, whereas an average of only 0.91% of propionyl erythromycin was excreted.

*Blood Partition.*—After oral administration of PELS, erythromycin was found to adsorb on, or penetrate, the red blood cells. After they were washed with saline, the red blood cells contained no detectable amount of erythromycin.

*Distribution.*—These results are summarized in Table V. In rats, a high concentration of erythromycin was found in the lungs, spleen, liver, kidneys, and heart. After administration of PELS, the erythromycin in the various tissues decreased more slowly than in tissues of rats receiving propionyl erythromycin. Brain tissue contained small amounts of erythromycin after administration of PELS. The amount of erythromycin in the brain was about 30 to 40% of the serum concentration.

*Absorption of PELS-S$^{35}$ and Sodium Lauryl Sulfate-S$^{35}$.*—Preliminary studies indicated that there was a very small amount of radioactivity in the blood following oral administration of PELS-S$^{35}$. Based on a blood volume of 5.18%, the total radioactivity in the circulation amounted to less than 1% of the dose. The total radioactivity in the liver and kidneys was even less. Most of the radioactivity was recovered in the gastrointestinal tract and some was excreted in the urine.

After oral administration of sodium lauryl sulfate-S$^{35}$, slightly more radioactivity was found in the blood, the liver, and the kidneys than in those tissues of rats receiving PELS-S$^{35}$. However, the radioactivity disappeared somewhat faster from tissues of rats given the first mentioned compound. Again most of the radioactivity was found in the gastrointestinal tract. Further studies are continuing.

TABLE II.—AVERAGE SERUM CONCENTRATION OF ERYTHROMYCIN (MCG./ML.) IN FOUR DOGS WITH DUODENAL FISTULA AFTER ADMINISTRATION OF PELS

| Route of Administration | Time in Hours | | | | | | |
|---|---|---|---|---|---|---|---|
| | ½ | 1 | 2 | 3 | 4 | 5 | 6 |
| Oral | <0.03 | 0.04 | 0.10 | 0.08 | 0.05 | 0.03 | 0.03 |
| Intraduodenal | 0.08 | 0.12 | 0.12 | 0.07 | 0.06 | 0.03 | 0.03 |

TABLE III.—BILIARY EXCRETION OF ERYTHROMYCIN IN ANESTHETIZED RATS[a] TWO HOURS AFTER ADMINISTRATION OF PELS

| Route of Administration | Serum Concn., mcg./ml. | Bile Volume, ml. | Bile Concn., mcg./ml. | % of Dose |
|---|---|---|---|---|
| Stomach | <0.03 | 1.78 (1.4–2.0) | 2.1 (1.5–6.0) | 0.02 |
| Duodenum | 0.073[b] (0.04–0.11) | 1.90 (1.5–2.4) | 6.8 (4.6–10.2) | 0.07 |

[a] The duodenum was ligated at the junction with the stomach.
[b] Numbers in parentheses are the individual ranges within the group.

TABLE IV.—BILIARY EXCRETION OF ERYTHROMYCIN IN RATS AFTER ORAL ADMINISTRATION

| Erythromycin | No. of Rats | Per Cent of Dose 0–7 hr. | 8–24 hr. | Total |
|---|---|---|---|---|
| PELS | 10 | 0.55 ± 0.07 | 0.99 ± 0.16 | 1.54 ± 0.17 |
| Propionyl erythromycin | 6 | 0.45 ± 0.10 | 0.46 ± 0.33 | 0.91 ± 0.17 |

## DISCUSSION

Rats which were fed PELS in concentrations equivalent to 50 mg./Kg./day for a period of three months showed normal weight gains, while doses of 100–500 mg./Kg./day produced some retardation of growth but no visceral changes attributable to the antibiotic. It is possible that the decreased food intake which accounted for the retardation of growth was a result of lack of palatability. Terminal serum

TABLE V.—TISSUE DISTRIBUTION OF ERYTHROMYCIN MCG./GM. OF WET WEIGHT

| | Propionyl Erythromycin | | PELS | |
|---|---|---|---|---|
| | 2 hr. | 7 hr. | 2 hr. | 7 hr. |
| Serum[a] | 0.62 (0.40–0.85)[b] | 0.30 (0.13–0.57) | 0.68 (0.52–1.00) | 0.41 (0.29–0.60) |
| Liver | 4.43 (2.3 –5.8) | 1.83 (0.9 –3.6) | 4.43 (3.0 –6.0) | 2.89 (2.0 –4.2) |
| Spleen | 4.79 (2.9 –6.6) | 2.76 (1.3 –4.6) | 4.30 (2.2 –6.3) | 3.29 (2.0 –5.6) |
| Kidneys | 3.59 (2.6 –4.0) | 1.11 (0.5 –2.1) | 3.07 (1.4 –4.4) | 1.91 (1.1 –3.1) |
| Lung | 7.50 (4.8 –10.8) | 3.54 (2.1 –5.4) | 5.79 (4.0 –7.2) | 5.53 (4.0 –6.8) |
| Heart | 2.36 (2.0 –3.6) | 0.74 (0.6 –1.1) | 3.34 (1.6 –5.5) | 1.55 (0.7 –2.6) |
| Brain | .. (0.00–0.16) | .. (0.00–0.05) | 0.20 (0.16–0.32) | 0.17 (0.12–0.28) |

[a] mcg./ml. [b] Numbers in parentheses are the individual ranges within the group.

concentrations show that the antibiotic was absorbed in proportion to the amount fed.

The changes in blood pressure, intestinal motility, and heart rate were very similar to those noted with erythromycin hydrochloride and propionyl erythromycin. It appears that these effects are due to the erythromycin content of the antibiotic.

In the rat, PELS produced better serum concentrations than propionyl erythromycin when equal amounts of antibiotic activity were given orally (Fig. 3). When equal weights of these two antibiotics were administered, higher serum concentrations were also obtained with PELS (Table V).

In rats, the major site of absorption of PELS is in the intestines. When PELS was administered to the stomach ligated at the junction with the duodenum, a small amount of erythromycin was recovered in the bile (Table III), indicating that the antibiotic was only slightly absorbed from the stomach. In dogs, PELS is also mainly absorbed from the intestines, since it produced earlier and slightly better serum concentrations after intraduodenal administration than after oral dose (Table II). We have observed (2) that erythromycin propionate was absorbed mainly in the intestines of both dogs and rats.

In anesthetized rats, the serum concentrations of erythromycin following PELS were much lower than the levels found in normal rats after similar doses. This indicates that both the anesthesia and the surgical trauma may have affected the gastrointestinal absorption of this antibiotic.

High concentrations of erythromycin were found in the various tissues following oral administration of both PELS and propionyl erythromycin (Table V). The highest concentration, 3.5 to 7.5 times that of the serum concentration, was in the lung. It is not known how much better PELS is absorbed from the gastrointestinal tract. However, its slower disappearance rate from the various tissues and its limited excretion through the bile may be factors in producing high serum concentrations of erythromycin.

Following oral administration of PELS, rats excreted about 1.54% of the dose in the bile in twenty-four hours (Table IV), about 64% of this amount being excreted during the period from the eighth through the twenty-fourth hour. It appears that this antibiotic is absorbed through the intestines for a relatively long period or that it is slowly released from the tissues to the circulation.

After administration of PELS erythromycin penetrated or was adsorbed on the red blood cells. On washing with saline, erythromycin disappeared from the red blood cells. This indicates that erythromycin is only loosely bound to the red cell component or loosely adsorbed on the cells, a factor beneficial to the therapeutic effectiveness of this antibiotic.

The superiority of propionyl erythromycin over erythromycin base when administered orally has been shown in rats by Lee, *et al.* (2), and in human subjects by Griffith, *et al.* (9). The ability of PELS to produce even better blood levels in rats has been reported in these studies. Data have also been presented that show the very low acute toxicity of this antibiotic, as well as its lack of pathogenic effects when administered daily over extended periods to rats and dogs.

Preliminary radioisotope studies with PELS-$S^{35}$ indicated that a significant amount of radioactivity was absorbed from the intestines. A large percentage of the radioactivity remained in the gastrointestinal tract. The quantity of lauryl sulfate in the highest concentration of PELS fed to rats (0.5%) amounts to approximately 0.15% in the diet. In a two-year study Fitzhugh and Nelson (10) have shown the lack of toxic effects from feeding sodium lauryl sulfate at a 1% level.

## SUMMARY

1. PELS was found to have very low acute toxicity. The determination of $LD_{50}$'s in mice, rats, and dogs was impractical from the standpoint of large quantity of drug needed and consequent mechanical problems..

2. Chronic studies have shown that rats fed PELS in their diet at a dose of approximately 50 mg./Kg./day gained weight comparable to normal animals. Higher concentrations resulted in some growth retardation. No visceral or hematopoietic damage was seen in any animals.

3. Dogs tolerated doses of PELS as large as 221 mg /Kg. (equivalent to 137 mg./Kg. of erythromycin activity) daily over extended periods.

4. Oral administration of PELS produced higher serum concentrations in rats than did propionyl erythromycin.

5. PELS, like the propionyl erythromycin, is mainly absorbed from the intestines of rats.

6. After oral administration of PELS, rats excrete the antibiotic in very small quantities in the bile.

7. High concentrations of erythromycin were found in the lungs, liver, spleen, kidneys, and heart after oral administration of PELS. Small amounts of erythromycin were also found in the brain..

8. It is believed that the superiority of PELS in producing high serum concentrations may be

partly due to its limited biliary excretion and its slow rate of disappearance from the various tissues.

9. Part of the blood erythromycin is loosely bound to red blood cell component or adsorbed on the cells.

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# Osmotic Concentration and Osmotic Pressure in Injectable Solutions*

By I. SETNIKAR and OLIMPIA TEMELCOU

**A method for determining the osmotic pressure of injectable solutions by measuring the variations of the red-cell volume is described. By means of this method, substances of pharmaceutical interest can be classified into different groups according to their diffusibility through the erythrocyte membrane and their action upon it. It was demonstrated *in vitro* and *in vivo* that for many substances the iso-osmotic concentration is not equivalent to the isotonic concentration and that the confusion between iso-osmia and isotonia can have dangerous consequences.**

IT IS COMMON KNOWLEDGE that only solutes which cannot pass through a barrier permeable to the solvent can exert an osmotic pressure (1); substances which can pass through the cell membranes cannot, therefore, counterbalance the osmotic pressure exerted by nondiffusible intracellular solutes.

Therefore, the osmotic concentration, measured by physical methods based on one of the colligative properties, is an expression of the osmotic pressure only when all the solutes present in solution are nondiffusible through the cell membranes, otherwise a solution found to be iso-osmotic is hypotonic for the cells.

This distinction would be of little practical importance were it possible to accept the view of Szekely and Goyan (2) to the effect that, of the substances in pharmaceutical use, those freely diffusible through the cell membranes are exceptional. The researches performed with a hemolytic method by Husa, *et al.* (3–8), demonstrate that, on the contrary, many substances in common pharmaceutical use, at a concentration iso-osmotic with blood, cause hemolysis for the very reason that they are unable to counterbalance the intracellular osmotic pressure.

While the hemolytic method can demonstrate very clearly the difference between solutes which are diffusible through the membrane of red cells and those which are not, it is not so easy to determine the isotonic concentration because, for this purpose, it is necessary to start from the premise that for all solutes there is a single ratio between isotonic concentration and hemolytic concentration, whereas it has been shown that, on the contrary, this ratio may vary from 1.4 to 3.1 (9).

Efforts have therefore been directed to the search for a method of direct determination of isotonic concentration and it has been found that this could be done fairly simply by means of a suitable modification of the hematocrit method used by Eijkman (10). This paper describes the method employed and the results obtained therefrom.

## METHOD

Human blood was drawn from a forearm vein; rabbit blood by cardiac puncture. The syringes used for drawing the blood were moistened with an 0.65% (isotonic) solution of NaF containing 5% heparin. The blood samples were centrifuged, the separated red cells were added to an equal volume of the solution under examination, and this suspension was centrifuged for thirty minutes at 3,000 r. p. m. in Wintrobe's hematocrit tubes. To determine the volume that the red cells would maintain in an isotonic solution, a similar test was performed mixing the red cells with the plasma of the same specimen of blood.

## RESULTS

**NaCl.**—The ratio between the volume of rabbit red cells and the concentration of NaCl is given in

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Fig. 1. In contact with plasma, red cells maintained a volume equal to that which they would take up in contact with an 0.93% solution of NaCl. This concentration is, therefore, isotonic with the red cell specimen used.

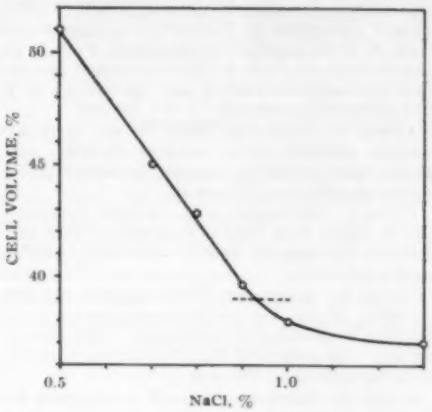


Fig. 1.—Effect of the NaCl concentration upon the volume of rabbit red cells. The dotted line represents the volume of the cells suspended in plasma (isotonic volume). For these red cells the isotonic concentration of NaCl was 0.93%.

**Urea.**—Solutions of urea from 1 to 2.6% (a 1.8% solution is iso-osmotic) caused complete hemolysis. The addition of NaCl at concentrations from 0.5 to 0.9% to an iso-osmotic solution of urea prevented laking, and the volume of the red cells was equal to that determined by solution of NaCl at the same concentration but without urea. Thus, urea does not of itself have a hemolytic effect, as in the opinion, for example, of Ebina (11); laking is brought about by the incapacity of this substance to counterbalance the intracellular osomotic pressure. In other words, as regards osmotic pressure, it is as if urea were not present in solution.

The incapacity of urea to exert an osmotic pressure can be demonstrated also *in vivo*. If 15 cc./Kg. of a 1.8% solution of urea is injected intravenously into rabbits, extensive hemolysis is observed, due to the destruction of about 3% of the red cells. A similar phenomenon is observed when one administers the same quantity of distilled water. Hemolysis can be entirely avoided by rendering the solution of urea isotonic with a 0.9% solution of NaCl.

**Dextrose.**—While dextrose exerts an osmotic pressure equal to its concentration on the erythrocytes of rabbits, on human erythrocytes the isotonic concentration is almost twice the iso-osmotic concentration (Fig. 2). The membranes of human erythrocytes would therefore seem to be partially permeable to dextrose. It is interesting to note that the resistance of human red cells increases in solutions of dextrose; the cell volume in hypotonic solutions can attain values practically twice those which usually precede hemolysis.

This increase in cell resistance perhaps accounts for the results obtained by Grosicki and Husa (4), who noted no difference between the hemolytic concentration of dextrose for human red cells and those for rabbit red cells.

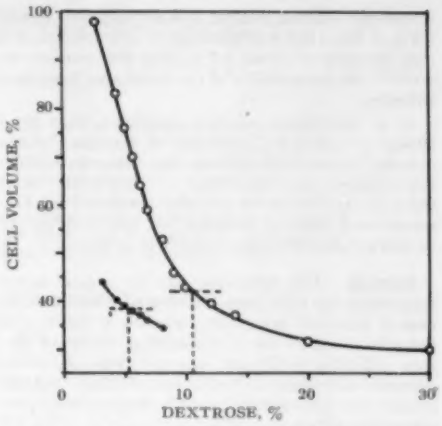


Fig. 2.—Effects of dextrose solutions at different concentrations upon the volume of the red cells of the rabbit and of man. The dotted line indicates the isotonic volume. ○—○, human red cells; ●—●, rabbit red cells.

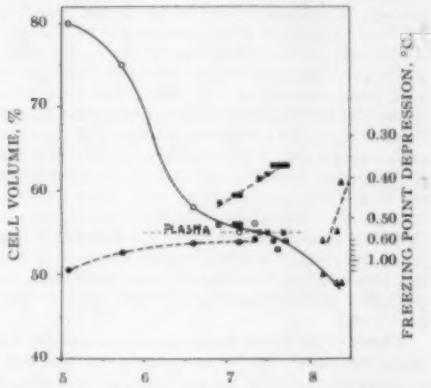


Fig. 3.—Effects of the pH upon the volume of red cells and thus also upon intracellular osmotic pressure. The cations concentration was maintained constant to 155 meq./L. of $Na^+$.

Left ordinate, cell volume; right ordinate, depression of the freezing point on a scale so adjusted that, for a solution of NaCl, the depressions of the freezing point would correspond to the cell volume; abscissa, pH of the buffer-red cell mixture.

The isotonic cell volume is shown by a dotted line. The straight line and the empty signs represents the cell volume, the dotted line and the full signs give the cryoscopic depression. ○— Buffers of acetic acid-sodium acetate, □— buffers of $NaH_2PO_4 - Na_2HPO_4$, △-buffers of barbital-sodium barbital. Note the poor correlation between the osmotic concentration measured by the cryoscopic method and the osmotic pressure exerted by the solutions upon the red cells.

**Procaine Hydrochloride.**—At its iso-osmotic concentration (5.05%), procaine hydrochloride causes hemolysis of the red cells of the rabbit. In contradistinction to what has been observed with urea, to

render the solution isotonic it is not sufficient to add 0.9% of NaCl but it is necessary to add this salt at a concentration of about 1.3%, as if the procaine increased the permeability of the membrane to sodium chloride.

It is interesting to note, however, that it is enough to add a 3.3% solution of dextrose (0.6 isoosmolar) to the same solution of procaine hydrochloride to have a solution which is isotonic for rabbit red cells, as if dextrose not only abolished the permeabilizing effect of procaine but rendered the cell membrane partially impermeable to procaine.

**Saponin.**—This substance has an intense hemolytic effect up to a concentration of 0.005–0.001% even if dissolved in an 0.9% solution of NaCl. The behavior of rabbit red cells placed in NaCl and dextrose solutions at different concentrations and in the presence of saponin at 0.05% was checked, and the results were very similar to those obtained with procaine hydrochloride at 5.05%.

**$ZnSO_4$.**—Zinc salts are of particular interest inasmuch as Hartman and Husa (6) observed by means of their hemolytic method that $ZnSO_4$ "protects" the red cells to such an extent that the isotonic solution of the salt would be 400 times more dilute than the iso-osmotic concentration. Cadwallader and Husa (12) have described similar results for zinc acetate. By our method, however, it can be shown that $ZnSO_4$ precipitates plasma proteins and causes hemolysis up to a concentration of 155 m*M*. The results described by Hartman and Husa can be confirmed only if blood and $ZnSO_4$ solution are mixed in the volumetric proportion of 1:50 (as these authors did), but it can also be demonstrated that the absence of laking is due to a precipitation and a denaturation of hemoglobin. One cannot, therefore, accept the conclusion that solutions of $ZnSO_4$ are isotonic at concentrations 400 times lower than the iso-osmotic concentration because not a protective action but a precipitating and denaturating action by the zinc ion is implicated.

**Effects of the pH of Solutions on Intracellular Osmotic Pressure.**—At physiological pH values negative charges prevail in red cell hemoglobin and about 50 meq./L. of cations are required for electrical neutralization. As hemoglobin is an ampholyte, its negative charges diminish when the environment becomes acid, releasing cations which, being unable to diffuse in the extracellular fluid through the cell membrane which is impermeable to them, attract anions from the extracellular fluid. A diminution of pH, therefore, involves a rise in intracellular osmotic pressure and, conversely, an augmentation of pH causes a fall in intracellular osmotic pressure. The isotonic concentration must, therefore, depend to some extent upon the pH of the solution.

Figure 3 gives experimental proof of this hypothesis. Although the cations concentration of the solution under examination was kept constant (155 meq./L. of $Na^+$), it may be observed that the cell volume increases in acid solutions and decreases in alkaline solutions, demonstrating that the osmotic pressure of red cells increases in contact with acid solutions and decreases in contact with alkaline solutions. Here again the osmotic concentration of the various solutions, measured by determining the depression of their freezing points, was not closely related to the osmotic pressure of the solutions.

## DISCUSSION

Similar experiments carried out on substances of pharmaceutical interest showed that these could be classified into the following groups:

Group 1.—Substances whose iso-osmotic concentration is isotonic: NaCl (0.9%), KCl (1.19%), sodium thiosulfate N. F. (2.98%), sodium borate U. S. P. (2.6%), sodium propionate N. F. (1.47%), sodium benzoate U. S. P. (2.25%), sodium barbital (3.14%), sorbitol (5.48%), and dextrose U. S. P. (5.5.%) for rabbit red cells.

Group 2.—Substances which do not exert any osmotic pressure: urea, succinic dinitrile, antipyrine, aminophylline, ethanol, propylene glycol, sodium pentobarbital, Tween 80.

Group 3.—Substances whose isotonic concentration is higher than their iso-osmotic concentration: dextrose (as regards human red cells), glycine, sodium salicylate.

Group 4.—Substances which increase the permeability of the erythrocyte membrane to NaCl: procaine hydrochloride, adiphenine hydrochloride, ethanol, and propylene glycol at higher than 10–20% concentrations, Tween 60.

Group 5.—Substances with a pronounced hemolytic action: saponin, sulfuric esters of methylandrostenediol and of testosterone.

Group 6.—Substances which exert a protective action similar to that exerted by dextrose as regards the increase in permeability caused by procaine: dextrose, sorbitol.

Group 7.—Substances precipitating proteins: $ZnSO_4$ and all precipitants of proteins.

## SUMMARY

When wishing to render an injectable solution isotonic, the main consideration should be the permeability of the cell membrane to the various solutes composing the solutions and the action of these solutes upon the cell membrane. In other words, *iso-osmia* (which can be determined by physical methods based on one of the colligative properties) is equal to *isotonia* only when all the solutes of the solution are unable to diffuse freely through the cell membrane. If this is not the case, isotonia can be determined only by measuring the osmotic effect of a given solution directly upon the concerned cells.

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# Polarography of Gibberellic Acid*

By WILLIAM F. HEAD, Jr.

**The polarographic characteristics of several gibberellins have been determined and a method for determining gibberellic acid in the presence of gibberellenic acid proposed. Gibberellic acid was found to possess a characteristic prewave in addition to a larger proton reduction wave shown by all gibberellins. This prewave may be measured with an accuracy to ± 2 per cent within definite concentration limits. Average recoveries of gibberellic acid from a typical formulation measured by the method was 97 per cent. The main reduction wave was shown to be that of carboxyl proton but the prewave reduction mechanism is not yet clear.**

THE FLUOROMETRIC ASSAY PROCEDURES used for gibberellic acid are dependent upon an effective separation of gibberellic acid from closely related compounds. Potassium carbonate chromatographic columns do not appear to accomplish this separation and other fluorescent, biologically inactive gibberellins are detected as gibberellic acid. The use of ultraviolet irradiation to suppress the fluorescent properties of gibberellenic acid also inhibits the gibberellic acid fluorophor. Recently, Kavanagh and Kuzel (1) have described a liquid partition system in which gibberellic acid is successfully separated from gibberellenic acid, a major interfering component.

The time-consuming biological assay (2) is absolute for plant growth activity but rather erratic results have been obtained.

In view of the isolation difficulties, a technique was sought which would allow measurement of gibberellic acid in small quantities in the presence of similar compounds. Kitamura and Sumiki (3) have pointed out that certain gibberellins are polarographically reducible, although no indication of specificity was given in their report.

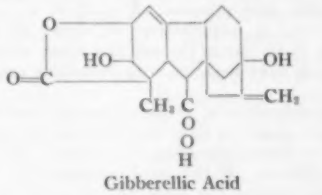
A mass isotope dilution assay based on the isolation of a pure sample of gibberellic acid and its isotope by crystallization has recently appeared (4). This method, advocated for fermentation broths, would not be conveniently applicable to samples containing only microgram quantities.

## EXPERIMENTAL

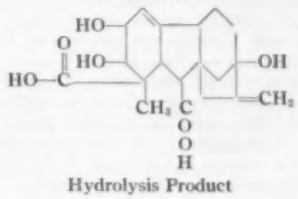
**Apparatus and Reagents.**—Most polarograms were made using a standard 20-ml. capacity H-cell with a dropping mercury electrode, agar-potassium chloride bridge, and a saturated calomel electrode. In some cases a quiet mercury pool was used as an anode in a single chamber cell. The polarograph used was a Sargent model XXI. All reagents used were analytical reagent grade. In the case of the quaternary ammonium supporting electrolytes, a solution of these reagents showed no polarographically reducible impurities over the voltage range examined.

**Polarographic Data.**—In Table I is listed the reduction characteristics of several gibberellins. All samples included were essentially pure products and a quantitative response was indicated for each reduction wave of all compounds. None of these compounds showed reduction waves from −2.0 to −2.7 volts (S. C. E.) when polarographed in a single cell with a quiet mercury pool anode in a 0.1 *N* tetraethylammonium chloride solution. The potentials listed in Table I were all taken in a 0.1 *N* potassium chloride supporting electrolyte.

The data for gibberellic acid were obtained with a working standard containing 94% gibberellic acid (by differential nonaqueous titration and differential fluorescence measurements), 1.5% gibberellenic acid (ultraviolet molecular extinction at 253 mμ = 19,200), and 4.5% moisture (Karl Fischer). Attempts were made to remove the gibberellenic acid by chromatography through potassium carbonate columns and subsequent elution with 1% water in acetonitrile. After two such treatments, the gibberellenic acid content was reduced to 0.28% and fluorescence in 85% w/w sulfuric acid indicated 99.3% gibberellic acid. When compared polarographically to the initial working standard a purity of only 75% was indicated. Infrared analysis, using the characteristic 5.67 μ lactone band of gibberellic acid and the working standard as a reference, indicated a gibberellic acid content of 82% in the "purified" material. It is thought that lactone hydrolysis may take place to an extent on carbonate columns with the hydrolysis product retaining the fluorescent characteristics of gibberellic acid.



Gibberellic Acid



Hydrolysis Product

* Received July 1, 1959, from the Analytical Research Department, Eli Lilly and Co., Indianapolis 6, Ind.

Dr. G. W. Probst and Dr. Koert Gerzon, who supplied the various gibberellins and much background information, are sincerely thanked for their help. A debt of gratitude is also due Dr. R. P. Miller and Mr. H. L. Bird, Jr., who chromatographed the various gibberellins during purification and performed the preparative chromatography work.

TABLE I.—REDUCTION POTENTIALS OF SEVERAL GIBBERELLINS

| Compound | $E_{1/2}$ (S. C. E.), Volts |
|---|---|
| Gibberellic acid | −1.0 to −1.2 |
| | −1.55 |
| Gibberellenic acid | −1.55 |
| Methyl ether of gibberellenic acid | −1.55 |
| Gibberic acid | −1.24 |
| | −1.54 |
| Tetrahydrogibberellic acid | −1.45 |

No attempts were made to isolate a hydrolysis product and the infrared data are the only evidence in support of such a reaction.

In a similar manner, attempts were made to purify gibberellenic acid from a sample containing 15% gibberellenic acid. After elution of gibberellic acid with acetonitrile containing 1% water, remaining gibberellins may be eluted with methanol. However, this technique resulted in a product containing only 46.7% gibberellenic acid when compared to the pure material at 253 mμ. The gibberellic acid polarographic wave at −1.0 to −1.2 v. (S. C. E.) was absent. Using the top phase of a 1:1 butanol-1.5 *M* ammonia mixture as the developing solvent on Whatman No. 1 paper, no other gibberellins could be detected with 0.5% potassium permanganate except gibberellenic acid. An attempt was made to purify gibberellenic acid by preparative chromatography in the above solvent system using the techniques of Brownell, Hamilton, and Casselman (6). The final purity of the nonmobile gibberellenic acid was 50.8%. This product had the same characteristics as the material purified using the column technique.

It is of interest that the 50% gibberellenic acid showed the same $i_d/c$ ratio at −1.55 v. (S. C. E.) on a weight basis as did the pure material. This points out the carboxylic acid nature of the impurities.

**Assay Principle.**—The polarograms of gibberellic and gibberellenic acid are shown in Figs. 1 and 2, respectively. It is apparent from Table I that the prewave of gibberellic acid is unique for the gibberellins except for gibberic acid. This material is a product of vigorous acid degradation of gibberellic acid (5) and is an unlikely interference in a fermentation broth or a commercial formulation. The gibberellic acid prewave at −1.0 v. to −1.2 v. (S. C. E.) is quantitative, as shown in Fig. 3, and its $E_{1/2}$ varies through the range mentioned, becoming more negative with increasing concentration.

Gibberellenic acid in the presence of gibberellic acid was shown to have no effect on this prewave. The two waves occurring at −1.55 v. (S. C. E.) were additive. If the total concentration of gibberellins became too large, fusion of the prewave and the additive waves would occur. Dilution would again separate the two waves; however, if gibberellic acid was present in very small quantities compared to total gibberellins, accurate measurement of the prewave would be difficult. Even with the 94% working standard gibberellic acid, fusion of the prewave can take place with its own reduction wave at −1.55 v. (S. C. E.) if the upper concentration limit of Fig. 3 is exceeded.

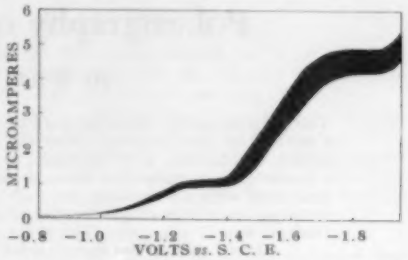



Fig. 1.—Gibberellic acid polarogram in 0.1 *N* KCl.

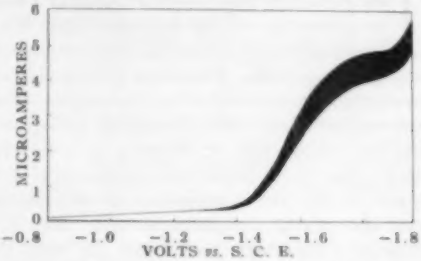



Fig. 2.—Gibberellenic acid polarogram in 0.1 *N* KCl.

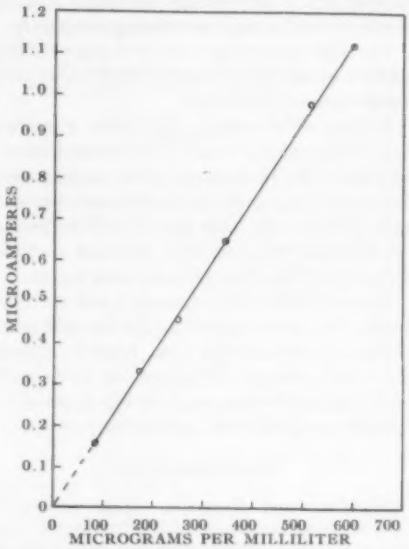



Fig. 3.—Gibberellic acid prewave current ($i_d$) *vs.* concentration.

Products such as tablets and bulk powders contain biologically inert materials which may interfere with a polarographic assay. Sodium lauryl sulfate and various dyes were found to be particularly offensive. The surface-active agent interfered seriously with the mercury drop rate as well as producing its own reduction wave in the region of the gibberellic acid prewave. The dyes, which can be used as maxima suppressors, completely prevented gibberellic acid reduction. In

a search for a selective solvent for gibberellins, redistilled methyl isobutyl ketone was found to be satisfactory even though traces of sodium lauryl sulfate were carried along with the gibberellins.

Crushed placebo tablet material was extracted in a beaker with redistilled methyl isobutyl ketone. The solvent was filtered, evaporated, and the residue dissolved in 0.1 *N* KCl. To this solution was added varying amounts of working standard gibberellic acid in order to establish a standard plot of $i_d/c$. It was necessary to use this procedure because the intercept did not occur at the origin due to the small amounts of sodium lauryl sulfate present. Using tablets of known gibberellic acid content, an average recovery of 97% was obtained by this method. The maximum deviation of the polarographic measurement was observed to be ± 2%.

## DISCUSSION

**Reduction Mechanism.**—A plot of $i_d/c$ for the reduction wave at −1.55 v. (S. C. E.) of gibberellic acid, shown in Fig. 4, immediately suggested a mechanism controlled by the ionization of the carboxyl group. Gibberellenic acid was observed to behave in a similar manner. These waves are within the range of compounds having reducible functions and one or more carboxylic acid groups.

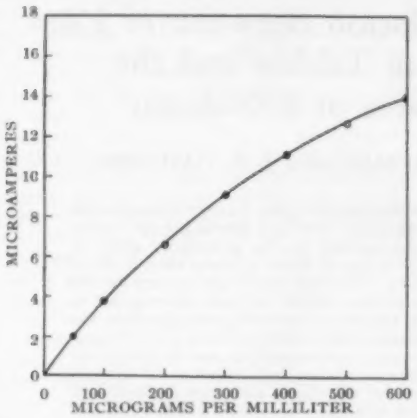



Fig. 4.—Concentration *vs.* $i_d$ using gibberellic acid wave at −1.55 v. (S. C. E.).

For confirmation of this assumption and in order to study the effects of pH on the prewave, a gibberellic acid solution in 0.1 *N* KCl was polarographed several times after adding small successive increments of 0.002 *N* NaOH. The procedure was repeated using 0.002 *N* HCl instead of alkali. As seen in Fig. 5, both waves of gibberellic acid decrease to zero μa. on addition of alkali. This zero intercept is equivalent to the titration endpoint of the solution. As would be expected, the wave at −1.55 v. (S. C. E.) increases steadily when the solution is made more acidic. The prewave, however, is relatively unaffected by acid as shown in Fig. 6. Both Figs. 5 and 6 are corrected for the effects dilution would have on $i_d$ and represent the effects of acid and alkali concentration only.

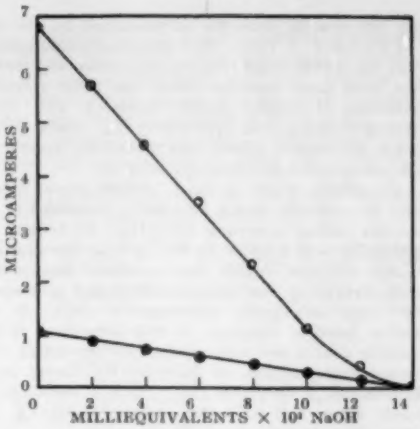



Fig. 5.—Effect of alkali on both reduction waves of gibberellic acid. ○ −1.55 v. (S. C. E.). ● −1.0 v. to −1.2 v. (S. C. E.).

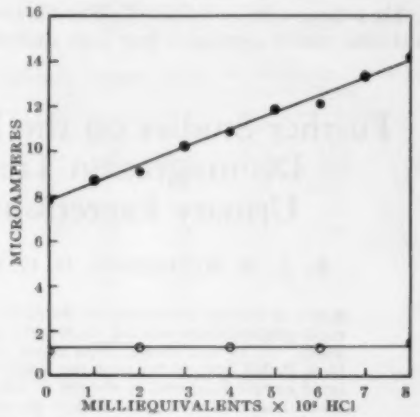



Fig. 6.—Effect of acid on both reduction waves of gibberellic acid. ● −1.55 v. (S. C. E.). ○ −1.0 v. to −1.2 v. (S. C. E.).

A satisfactory explanation of the mechanism of reduction of the prewave is more difficult to obtain. Approximation of the Ilkovic equation gave a value for *n* (apparent electron change per molecule reduced) of 0.38 while plots of log $i_d - i/i$ *vs.* $E_{1/2}$ gave a value of 0.44. This points up the irreversibility of the reduction but the polarographic wave slope is too large to be accounted for by an association or polymerization mechanism (7).

On examination of the structure of gibberellic acid, the only other apparent reducible sites are the methylene group, the lactone group, and the double bond. Since gibberellenic acid contains the same methylene group, it would not be consistent to assign the prewave of gibberellic acid to reduction of this group. A similar case may be drawn for the lactone group. Tetrahydrogibberellic acid contains the same lactone group as gibberellic acid and is a completely saturated compound. It does not show the prewave reduction. In addition, lactones

are not usually reducible at potentials as low as −1.2 v. (S. C. E.) (8). By a process of elimination, only the double bond remains as a choice and there has been some question about the polarographic reduction of isolated double bonds (9, 10). Dihydrogibberellic acid (gibberellin $A_1$) would offer much information about this reduction; however, this compound is not presently available.

The effects of pH on the $i_d$ of both waves show that the prewave is not kinetically controlled in a manner similar to pyruvic acid (11). By heating a gibberellic acid solution to 80°, a large increase in $i_d$ was obtained, rather than complete disappearance, indicating that the prewave is not produced by some adsorption phenomenon (12). If a double bond is involved in this reduction, it is possible that a two-step mechanism dependent on proton concentration, as discussed by Elving and Teitelbaum, is operative (13). Such a mechanism would agree well with the observed behavior of gibberellic acid reduction in alkaline media.

## SUMMARY AND CONCLUSIONS

The polarographic properties of gibberellic acid and some related compounds have been studied. Gibberellic acid reduction is shown to consist of two separate mechanisms giving rise to a normal wave and a prewave. This prewave was found in only one other gibberellin examined and an assay method was based on this characteristic.

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# Further Studies on the Relation Between *in Vitro* Disintegration Time of Tablets and the Urinary Excretion Rates of Riboflavin*

By A. B. MORRISON, D. G. CHAPMAN, and J. A. CAMPBELL

**Rates of urinary excretion of riboflavin were determined after dosing subjects with eight preparations varying in *in vitro* disintegration time and physiological availability. *In vitro* disintegration times were determined by the procedure given in U. S. P. XV Second Supplement, modified by the use of thirty minutes time in simulated gastric fluid and by the use of solid disks. Previous work was confirmed and additional evidence presented to show that tablets which did not disintegrate in one hour by this procedure were not fully available to the body, as judged by the urinary excretion of riboflavin. Use of the fluted disks of the existing U. S. P. procedure did not change the relationship between *in vivo* availability and *in vitro* disintegration time. The height of riboflavin excretion curves was found to be directly proportional to physiological availability of the riboflavin and was inversely related to *in vitro* disintegration time. Since rate of urinary excretion has been shown to be directly related to tissue levels for several drugs, it might be expected that marked differences in blood levels of riboflavin would be produced by the various preparations tested.**

THE RESULTS of earlier studies from this laboratory (1–3) suggested that tablets which did not disintegrate in one hour by a specified *in vitro* test were not fully available to the body, as judged by the urinary excretion of doses of riboflavin and *p*-aminosalicylate. Although Endicott and Kirchmeyer (4) questioned these observations, their findings that the riboflavin in tablets disintegrating in thirty-two to forty-one minutes was fully available *in vivo* are in complete agreement with those of Chapman, *et al.* (1). Vliet (5) suggested that some of the tablets which Chapman, *et al.* (1), found to have *in vitro* disintegration times of over sixty minutes, may have been enteric coated. In view of these questions, the present studies were conducted to investigate more closely the relationship between availability of riboflavin *in vivo* and the *in vitro* disintegration of sugar coated tablets, particularly those disintegrating within sixty to one hundred and twenty minutes.

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The authors are grateful for the continued cooperation and assistance of the subjects who took part in this investigation, and are indebted to Mr. L. G. Chatten for some of the *in vitro* disintegration times reported, and to Mr. C. Perusse for chemical determinations.

It has been established that for several drugs, including sulfaethylthiadiazole (6), creatinine (7, 8), xylose (9) and galactose (10), the rate of urinary excretion is proportional to tissue concentration. Since the rate of urinary excretion of riboflavin is probably also related to tissue concentration, data were obtained on variation in excretion rate over a period of time after dosing with the various riboflavin-containing preparations.

## METHODS

**Physiological Availability.**—As in previous studies (1, 2), the method used to determine the physiological availability of riboflavin was essentially that of Melnick, *et al.* (11). Eight sugar coated products were tested during a two-year period. Six to 9 normal male subjects, shown to be receiving nutritionally adequate diets, were used in the experiment. While on test, they were allowed to consume their regular meals but were cautioned to refrain from eating foods high in riboflavin, such as liver, and to eat meals similar in nature from day to day. The subjects were given doses of 5 mg. or 10 mg. of riboflavin in a rapidly disintegrating standard tablet, and 3 mg. to 10 mg. of riboflavin as the commercial preparations. The doses were given at 8:45 a. m. after breakfast. Urine was collected in opaque bottles containing 2 ml. of 3.5 *N* $H_2SO_4$ at two-, four-, six-, eight-, fourteen-, and twenty-four-hour intervals after dosing. Riboflavin in the tablets and urine was determined by the U. S. P. XV fluorometric procedure (12). All gross excretion values were corrected by subtracting the appropriate blank determined on the urine of the same subjects without dosing. Calculations of availability were based on duplicate determinations of riboflavin in the tablets.

**In Vitro Disintegration Times.**—The apparatus described in U. S. P. XV Second Supplement (13), modified by the use of solid plastic disks, was used to determine disintegration time. The tablets were immersed for thirty minutes in simulated gastric fluid and the remainder of the time in simulated intestinal fluid. Disintegration times reported were mean times of at least two separate tests on six tablets each.

## RESULTS

Descriptive data on the preparations used are summarized in Table I. All of the products were sugar coated multivitamin preparations. Products B, C, E, F, and H also contained minerals. *In vitro* disintegration times varied from sixty-two minutes (product H) to one hundred and twenty minutes (product D).

The data on urinary excretion of riboflavin after ingestion of 5 mg. or 10 mg. of the standard dose are summarized in Table II. The mean excretion in five trials conducted over a two-year period varied from 57 to 60%, with an overall mean of 58%. As might be expected in view of the findings of Melnick, *et al.* (11), Hegsted, *et al.* (14), and others, considerable variation was found in the percentage of the standard dose of riboflavin excreted by the various subjects. Significant variation in percentage excretion by the same individual in different trials was also noted. The remarkable constancy in the mean percentage excretion observed in repeated trials in the present studies is in agreement with the findings of Melnick, *et al.* (11).

The results of the physiological availability studies are summarized in Table III. The high

TABLE I.—DESCRIPTION OF PRODUCTS STUDIED

| Product | Medication | *In Vitro* Disintegration Time, min. | Riboflavin/Unit, mg. | Riboflavin/Dose, mg. |
|---|---|---|---|---|
| A | Multivitamins | 69 | 6.0 | 6.0 |
| B | Multivitamins and minerals | 75 | 5.0 | 10.0 |
| C | Multivitamins and minerals | 75 | 5.0 | 5.0 |
| D | Multivitamins | 120 | 4.0 | 4.0 |
| E | Multivitamins and minerals | 78 | 2.5 | 5.0 |
| F | Multivitamins and minerals | 69 | 2.5 | 5.0 |
| G | Multivitamins | 69 | 2.0 | 4.0 |
| H | Multivitamins and minerals | 62 | 3.0 | 3.0 |

TABLE II.—PERCENTAGE OF STANDARD DOSE OF RIBOFLAVIN RECOVERED IN URINE

| Subject | Trial 1 10-mg. Dose 1957 | Trial 2 5-mg. Dose 1957 | Trial 3 5-mg. Dose 1959 | Trial 4 5-mg. Dose 1959 | Trial 5 5-mg. Dose 1959 |
|---|---|---|---|---|---|
| A. B. M. | .. | .. | 36 | 52 | 72 |
| J. A. C. | 58 | 56 | 66 | 64 | 54 |
| T. K. M. | 38 | 51 | 53 | .. | 75 |
| J. M. M. | 53 | 49 | 75 | .. | 62 |
| R. C. | 78 | 76 | .. | .. | .. |
| D. G. C. | 63 | 62 | .. | .. | .. |
| L. C. | 56 | 41 | .. | .. | .. |
| T. P. | 56 | .. | .. | 79 | .. |
| E. M. | 60 | 67 | .. | .. | .. |
| O. P. | 60 | .. | .. | .. | .. |
| C. P. | .. | .. | 52 | 27 | 50 |
| D. S. | .. | .. | 74 | 50 | 49 |
| K. S. | .. | .. | 51 | 67 | 59 |
| Mean | 58 | 57 | 58 | 57 | 60 |

TABLE III.—PHYSIOLOGICAL AVAILABILITY OF RIBOFLAVIN IN EIGHT PRODUCTS

| Subject | Riboflavin Available in Product, % A | B | C | D | E | F | G | H |
|---|---|---|---|---|---|---|---|---|
| A. B. M. | ... | .. | .. | ... | 33 | 88 | 11 | 66 |
| J. A. C. | 103 | 36 | 14 | 5 | 80 | 97 | 35 | 91 |
| T. K. M. | ... | 27 | 0 | 11 | 12 | 54 | 38 | 58 |
| J. M. M. | ... | 80 | 2 | 0 | 69 | 120 | 92 | 98 |
| R. C. | 81 | 65 | 48 | 8 | .. | ... | .. | .. |
| D. G. C. | 46 | 52 | 98 | 0 | .. | ... | .. | .. |
| L. C. | ... | 39 | 2 | ... | .. | ... | .. | .. |
| T. P. | 42 | 56 | .. | ... | .. | ... | .. | 79 |
| E. M. | 98 | 23 | 2 | 22 | .. | ... | .. | .. |
| O. P. | 62 | 18 | .. | 53 | .. | ... | .. | .. |
| C. P. | ... | .. | .. | ... | 19 | 139 | 42 | .. |
| D. S. | ... | .. | .. | ... | 77 | 53 | 51 | .. |
| K. S. | ... | .. | .. | ... | 64 | 14 | 45 | 21 |
| Mean | 72 | 44 | 24 | 14 | 51 | 81 | 45 | 69 |
| *In vitro* disintegration time | | | | | | | | |
| Solid disks | 69 | 75 | 75 | 120 | 78 | 69 | 69 | 62 |
| Fluted disks | ... | .. | .. | ... | 89 | 84 | 61 | 60 |

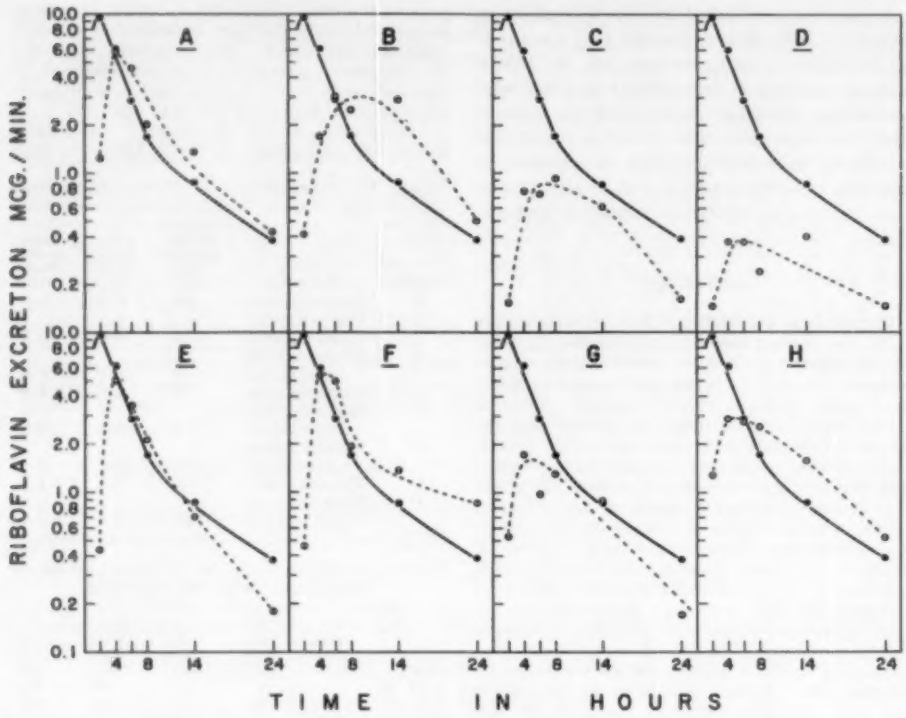


Fig. 1.—Urinary excretion curves of riboflavin in eight tablets (○- - -○) disintegrating *in vitro* in one to two hours compared with curves for a riboflavin standard tablet (●——●).

variation noted between subjects is typical of results obtained with preparations on the borderline of availability (1). None of the tablets was fully available physiologically. *In vitro* disintegration times tended to be inversely related to physiological availability. Preparation A, which disintegrated in sixty-two minutes *in vitro*, was only 69% available *in vivo*. Preparation D, which had the longest disintegration time studied (one hundred and twenty minutes), was the least available *in vivo* (14%). The riboflavin in this preparation was completely unavailable to two of the seven subjects who consumed it.

Data on rate of excretion of riboflavin after ingestion of the eight preparations were compared with those for the combined 5-mg. standards in Fig. 1. A semilog plot was used, as suggested by Swintosky, *et al.* (6). The peak urinary excretion rate declined with increasing disintegration time and was directly related to physiological availability. The peak of riboflavin excretion occurred earlier with the standard than with any of the preparations studied. The maximum rate of urinary excretion (0.40 mcg./min.) found with the product of lowest physiological availability (product D) was only 4.2% of that found with the standard (9.45 mcg./min.). The excretion curve found with product A was similar in shape to that found with the standard, except that the highest excretion rate recorded was only 5.92 mcg./min. as compared to 9.45 mcg./min. for the standard. Products B, C, and D showed low peak excretion rates and low physiological availability. Product E, which was 51% available *in vivo*, showed a peak excretion rate of 5.15 mcg./min. However, the rate of excretion found with this product declined somewhat more rapidly than did that of the standard. The peak excretion rate attained with product F was only 5.48 mcg./min., but the riboflavin in this product was excreted more slowly than that in the standard dose, resulting in the highest availability (81%) of the products tested. Products G and H had peak excretion rates of only 1.71 and 2.89 mcg./min., respectively.

In further studies, the details of which are not reported herein, the physiological availability of riboflavin was determined in two pelleted "timed release" preparations containing amphetamine and vitamins and prepared by the same manufacturer. The riboflavin in one preparation was fully available *in vivo*, whereas that in the second preparation was only 36% available. *In vitro* disintegration times could not be determined on these pelleted products by the U. S. P. procedure.

*In vitro* disintegration times on four of the preparations studied herein were also determined by use of the fluted disks described in U. S. P. XV Second Supplement (13). The results were plotted against percentage physiological availability in Fig. 2. For comparison, the data of Chapman, *et al.* (1), obtained with rubber disks, and those of the present studies obtained with solid plastic

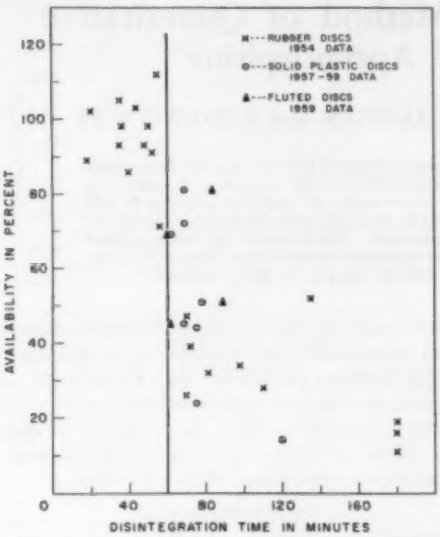


Fig. 2.—Relation between *in vitro* disintegration time of sugar coated tablets and physiological availability of riboflavin.

disks, were also plotted. The relationship found between *in vitro* disintegration time and *in vivo* availability was similar with all three types of disks, i.e., of 33 different tablets tested, none with *in vitro* disintegration times exceeding sixty minutes were fully available *in vivo*.

## DISCUSSION

The results of the present studies confirm those published previously (1–3) and would appear to remove any doubt there may have been concerning the validity of the sixty-minute time limit suggested for the *in vitro* disintegration of sugar coated tablets containing riboflavin. A disintegration time of sixty minutes obtained by the procedure used herein would be approximately equivalent to ninety minutes by the U. S. P. procedure. It has recently been proposed that the time limit for decavitamin tablets be reduced from four hours to two hours (15). The results of the present studies would indicate that the riboflavin in sugar coated tablets requiring two hours for *in vitro* disintegration by the U. S. P. test would not be fully available to many subjects. It is obvious that the one-hour time limit for tablets containing riboflavin only applies to those tablets which disintegrate uniformly. It does not hold for tablets in which the riboflavin is on the outside of a relatively resistant core, or beside a more resistant layer. It is still possible of course, as Endicott and Kirchmeyer (4) have pointed out, that a somewhat different relationship might be obtained with other vitamins which are more soluble than riboflavin. This point is largely academic, however, since most multivitamin tablets contain riboflavin and if a longer disintegration time were permitted, the riboflavin content of such tablets would not be available to the body.

As was pointed out previously (16) the use of urinary excretion data to evaluate the release of drugs requires recognition of the fundamental relationship that exists between concentration or amount of drug in the blood and other fluids of distribution, on the one hand, and excretion rate of the drug on the other. It has been established both by experimental work and theoretical considerations that for several drugs, the excretion rate is directly proportional to the amount or concentration of drug or other exogenous substances in the blood. This relationship has been shown to be true for creatinine, xylose, and penicillin. There is considerable evidence (17) that, in human subjects, the rates of urinary excretion of water-soluble vitamins are directly proportional to tissue levels and to the quantity of vitamins consumed, if the subjects are subsisting on an adequate diet. There seems little doubt, therefore, that measurement of the rate of urinary excretion of riboflavin, after dosing normal subjects with various riboflavin preparations, provides a valid measure of the availability of the dose to the body and of tissue levels produced by the dose. Thus, marked differences in blood levels of riboflavin might be expected after ingestion of the products tested in the present studies. For instance, it seems reasonable to assume that the blood level of riboflavin found with product D (14% available *in vivo*) would at no time be more than approximately 5% of that found with the standard. It is obvious that such a product would be unlikely to yield desired clinical effects.

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# A Rapid and Sensitive Method of Quantitative Determination of Apomorphine*

By P. N. KAUL, E. BROCHMANN-HANSSEN, and E. LEONG WAY

**A rapid and specific assay procedure has been developed for the routine determination of apomorphine. The method involves oxidation of the compound by mercuric chloride and ultraviolet absorption analysis of the extracted reaction product at 330 mμ. The product obeys Beer's law over a wide range of concentration. Optimum conditions for the reaction have been investigated. The method has been applied to the recovery of added apomorphine from aqueous solutions and biological materials. As little as 0.1 mcg. per ml. can be assayed by the procedure.**

APOMORPHINE exhibits unique pharmacological properties when administered to different animal species. Although used clinically as an expectorant and emetic, its usage for stimulating race horses has also been suspected. Detection of apomorphine, illegally administered to such animals, and studies on its metabolism require a sensitive and specific method for its identification and quantitation.

Although several more or less specific color reactions have been developed for qualitative detection of apomorphine (1, 2), no satisfactory method is available for its quantitative determination in microquantities. Titration procedures in aqueous or nonaqueous media (3, 4) are not sufficiently sensitive or specific for most biological work. General phenol reactions, adapted to quantitative determination of apomorphine (5), also lack specificity.

Apomorphine is a strong absorber of ultraviolet radiation, and it can, if of sufficient purity, be determined by ultraviolet spectrophotometry (6). However, because of its labile nature, it has proved difficult to purify this compound to the extent that a low blank value may be obtained from biological materials, without appreciable loss of apomorphine.

In 1915, Grimbert and Leclere (7) reported a color reaction for apomorphine based on its oxidation by mercuric chloride in a weakly basic medium of sodium acetate. The oxidation product was extracted with amyl alcohol which acquired a blue color. This reaction is used as a test for identity of apomorphine in the French Pharmacopeia (8), and is said to have a sensitivity of about 2 p. p. m.

In this paper, the original color reaction of Grimbert and Leclere has been studied, modified to further increase its sensitivity, and adapted to quantitative determination of apomorphine. The resulting method has been found to be of particular value for analysis of microquantities of the compound in biological materials because naturally occurring catecholamines, related structurally to apomorphine, do not interfere.

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## EXPERIMENTAL

**Reagents and Equipment.**—Saturated mercuric chloride solution: 5 Gm. of mercuric chloride is dissolved in 100 ml. of water by heating on a steam bath, the solution is allowed to cool to room temperature, and filtered; isoamyl acetate, Mallinckrodt A R grade; McIlvaine buffer solution, pH 6.0 (9); apomorphine hydrochloride, U. S. P.; 50-ml. glass-stoppered centrifuge tubes; a suitable pH meter, Leeds Northrup model was used; and a suitable spectrophotometer for recording absorbance at 330 mμ. The Cary model 11 recording instrument was used for the work reported here.

**Analytical Method.**—The solution to be analyzed is diluted to a concentration of about 15 to 30 mcg. per ml. of apomorphine. To a 50-ml. glass-stoppered centrifuge tube are added 4 ml. of the diluted apomorphine solution, 1 ml. of the buffer solution, and 3 drops of the mercuric chloride solution. The mixture is heated in a water bath at 70° for three to four minutes, and then cooled to room temperature under running water. Ten milliliters of isoamyl acetate is added with a pipet and the tube shaken for one minute. The phases are separated by centrifugation, and the absorbance of the isoamyl acetate layer determined at 330 mμ against the solvent. The amount of apomorphine in the 4-ml. aliquot is obtained from a standard curve prepared from known solutions analyzed in the same way.

**Spectral Characteristics.**—Figure 1 shows the absorption spectrum of the colored reaction product in isoamyl acetate. The oxidation product exhibits two maxima at 540 mμ and 400 mμ in the visible region, and a sharp maximum of much greater absorptivity at 330 mμ. In amyl alcohol, the extraction solvent originally suggested by Grimbert and Leclere (7), the absorptivities are only about one-half of the corresponding values in isoamyl acetate. It is interesting to note that although the reaction is a colorimetric identification test for apomorphine, the absorbance measurement at 330 mμ in the ultraviolet region serves as a sensitive means for its quantitative estimation. The reagent blank at this wavelength reads zero absorbance.

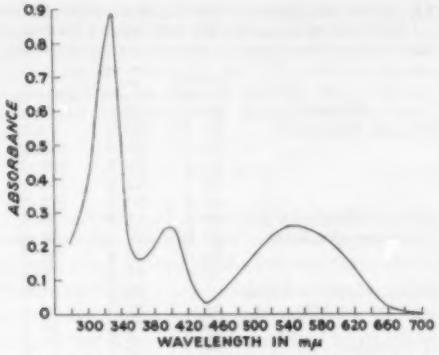


Fig. 1.—Absorption spectrum of the oxidation product of apomorphine in isoamyl acetate.

The oxidation product exhibits different colors in different solvents, perhaps due to the formation of different π-complexes. The color is green in water; blue in methanol, ethanol, amyl alcohol, and chloroform; pink in benzene, ether, and carbon tetrachloride; and violet in isoamyl acetate. In all these solvents, the peak at longer wavelength in the visible range shows a considerable shift, whereas there is only a slight shift in the ultraviolet peak.

**Effect of pH.**—The oxidation was carried out as described under "Analytical Method" at 70° for four minutes, while the pH was varied from 2.2 to 8.0 with McIlvaine buffers. The effect of pH on the reaction is illustrated in Fig. 2. Maximum absorbance values are obtained at pH 6.

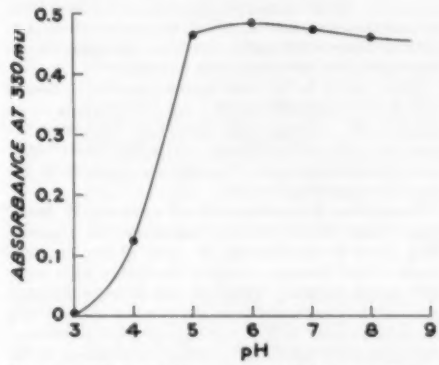


Fig. 2.—Effect of pH on the oxidation reaction of apomorphine.

**Effect of Temperature and Time.**—Figure 3 shows the temperature dependence of the reaction at a constant pH of 6.0 and a heating time of six minutes. The combined effect of time and temperature on the reaction is illustrated in Fig. 4.

At room temperature the reaction is slow, but the rate increases rapidly with an increase in temperature. Although various time-temperature combinations above 60° will give satisfactory results, a heating time of three to four minutes at 70° was chosen as most convenient. At lower temperatures, the reaction never reaches an optimal maximum. Prolonged heating results in decomposition of the reaction product, more so, however, at lower than at higher temperatures.

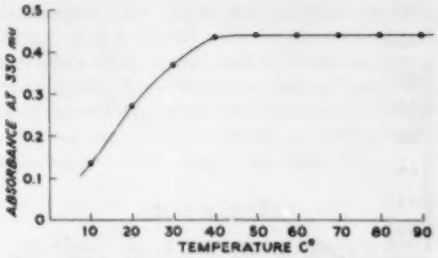


Fig. 3.—Effect of temperature on the oxidation reaction of apomorphine.

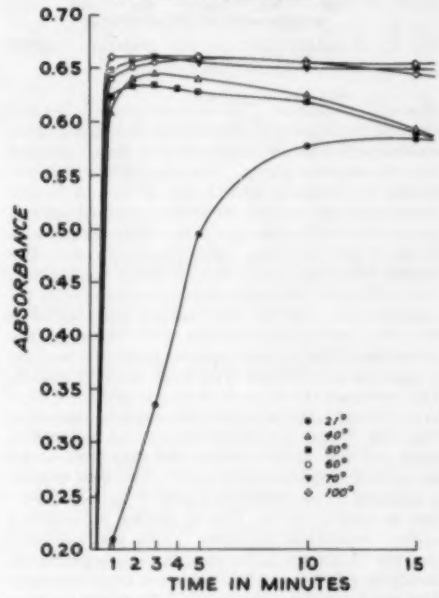


Fig. 4.—Effect of time of heating of the reaction mixture at different temperatures. Absorbance readings at 330 mμ.

**Effect of Concentration.**—The effect of concentration of apomorphine on the reaction is given in Fig. 5, which shows that the reaction obeys Beer's law over a wide range.

**Specificity.**—The color reaction was carried out with catechol, epinephrine, norepinephrine, and dihydroxyphenylalanine (DOPA). None of the colored reaction products given by these substances showed any absorbance at 330 mμ.

**Procedure for Biological Materials.**—While the analytical method described above is directly applicable to simple apomorphine preparations, such as injections or tablets, analysis of more complex preparations or biological fluids requires an extraction and purification step prior to the actual determination. The extraction procedure is: to a 4-ml. aliquot of diluted preparation in a separatory funnel are added enough 10% ammonia solution to give a pH of 6 to 7 and 20 ml. of C. P. ethyl acetate. The mixture is shaken for two minutes and the phases

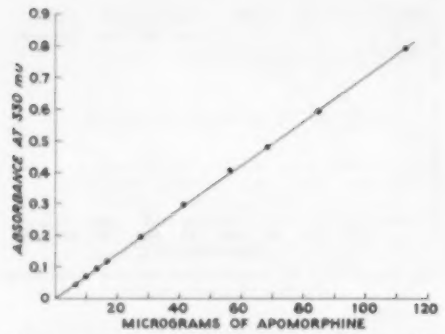


Fig. 5.—Standard curve for the oxidation reaction of apomorphine.

allowed to separate. The aqueous phase is drained into another separatory funnel and the organic phase washed with 1 ml. of water which is then combined with the aqueous phase. The ethyl acetate layer is shaken immediately with 5 ml. of 0.05 *N* hydrochloric acid and set aside while the combined aqueous layer and wash from the first extraction is shaken with 10 ml. of ethyl acetate. After separation of the two phases, the ethyl acetate layer is again washed with 1 ml. of water, the wash being combined with the aqueous layer and the ethyl acetate layer added to the ethyl acetate-hydrochloric acid mixture. The extraction of the original aqueous phase and washing is repeated a third time with 5 ml. of ethyl acetate. The combined ethyl acetate extracts and the 0.05 *N* hydrochloric acid are shaken for about two minutes. The acid extract is collected in a 10-ml. volumetric flask, and the extraction repeated with 3 ml. and 2 ml. of 0.05 *N* hydrochloric acid. The final volume is adjusted to the mark with acid. The color reaction is carried out on a 4-ml. aliquot as described under "Analytical Methods," with the exception that the McIlvaine buffer used in these experiments should be pH 6.4 and five times the usual strength. This produces the desired pH of 6.0 in the reaction mixture.

**Addition and Recovery Experiments.**—Known amounts of apomorphine hydrochloride were dissolved in acidified water and urine specimens. Aliquots of 4 ml. were subjected to the extraction procedure described above, and finally assayed by the "Analytical Method." Table I gives the per cent recoveries from aqueous solution and urine specimens of humans, horses, and rabbits. It can be seen that while the recovery from urine was about 20% lower than that from aqueous solution, the respective recovery values from the urine of each species were quite consistent, varying less than ±3%. Zero absorbance at 330 mμ was observed when the blank specimens of all species were carried through the procedure.

## DISCUSSION

Apomorphine is fairly stable in acidic aqueous medium, but its stability decreases rapidly with increasing pH. The addition of ascorbic acid has been used to stabilize apomorphine preparations (10). In our work the use of ascorbic acid to ensure greater recoveries of added apomorphine from biological fluids was of no value. Solvents also seem to have an effect on the stability of the free base. Chloroform favors a rapid decomposition, whereas ethyl acetate has been found to be the most satisfactory solvent for immiscible solvent extraction. It effects complete extraction of the base from an aqueous solution at pH 6 to 8, and if shaken with aqueous acid within three to five minutes, no detectable loss of apomorphine results.

Table I.—Recoveries of Apomorphine After Addition to Aqueous Solutions and Urine

| Material | Amount Added, mcg. | Amount Recovered, mcg. | Recovery, % |
|---|---|---|---|
| Aqueous Solutions | 84.5 | 83.0 | 98.2 |
| | 84.5 | 83.8 | 99.1 |
| | 84.5 | 84.5 | 100.0 |
| | 84.5 | 82.8 | 98.1 |
| | 84.5 | 86.4 | 102.2 |
| Human urine with added ascorbic acid | 86.6 | 67.8 | 78.4 |
| | 86.6 | 69.8 | 80.6 |
| | 86.6 | 70.8 | 81.8 |
| | 86.6 | 67.8 | 78.4 |
| Human urine without added ascorbic acid | 85.5 | 72.0 | 84.5 |
| | 85.5 | 71.0 | 83.4 |
| | 85.5 | 72.2 | 84.6 |
| Horse urine | 20.9 | 16.2 | 77.5 |
| | 20.9 | 16.5 | 78.7 |
| | 20.9 | 16.5 | 78.7 |
| | 20.9 | 16.5 | 78.7 |
| | 20.9 | 16.5 | 78.7 |
| | 31.4 | 24.9 | 79.3 |
| | 31.4 | 25.3 | 80.6 |
| | 31.4 | 24.9 | 79.3 |
| | 31.4 | 26.0 | 82.8 |
| Rabbit urine | 95.5 | 78.8 | 82.5 |
| | 95.5 | 78.0 | 81.7 |
| | 95.5 | 79.5 | 83.3 |
| | 95.5 | 78.5 | 82.2 |

The type of buffer used to maintain the optimum pH of the oxidation reaction mixture appears to be immaterial. Acetate and phosphate buffers of the same pH give similar results. The McIlvaine buffer was preferred because of its greater capacity in the desired range of pH ± 0.2.

Recoveries from urine were consistently lower than those from aqueous solutions. It appears that there is modification of some of the apomorphine either through complex formation with some substances normally found in the urine or because some of the drug is strongly adsorbed on the colloidal material present in it. Various attempts to recover the apparently modified or adsorbed fraction of the drug through (*a*) the hydrolysis of the residual urine left after extraction procedure and then re-extraction of the liberated apomorphine, if any, (*b*) the use of modified extraction procedure, involving excessive immiscible solvent extractions, and (*c*) modifying the conditions for the color reaction to improve the recovery, were all in vain. Further work along these lines to explain the discrepancy is in progress.

Relatively high specificity of the reaction for apomorphine and insignificant blank absorption values obtained from biological materials when carried through the procedure indicate the usefulness of the method for biological work. By increasing the path length of absorption and reducing the extraction volume of isoamyl acetate, the sensitivity

of the method may be increased many-fold. As little as 0.1 mcg. per ml. of apomorphine can be easily estimated. Such a sensitivity would find application in studying the *in vitro* enzymatic degradation of the drug. Presently, the method is being used for studying the metabolism of apomorphine.

## SUMMARY

The oxidation of apomorphine hydrochloride with mercuric chloride under standard conditions gives a green-colored product which turns violet when extracted into isoamyl acetate. The absorption peak at 330 mμ exhibited by the colored reaction product, in addition to its peaks in the visible region, has been used for quantitative determination of apomorphine. Catecholamines and other related polyhydroxyphenols in urine do not interfere in the estimation.

Optimum conditions for the reaction have been determined. By simple manipulations, as little as 0.1 mcg. per ml. can be determined. A procedure has been described for the extraction of apomorphine from biological material and solutions containing interfering substances, and the color reaction has been applied to such extracts from human, horse, and rabbit urines.

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# Kinetics of the Specific Base-Catalyzed Hydrolysis of Naphazoline*

By MARVIN J. STERN†, LOUIS D. KING, and ARNOLD D. MARCUS‡

**The rate of the specific base-catalyzed hydrolysis of naphazoline has been found to be first order with respect to both naphazoline and hydroxyl ion, regardless of whether the naphazoline exists as the protonated or unprotonated species. A theoretical isothermal equation expressing the pseudo-first order specific rate constant as a function of the hydronium ion and hydroxyl ion activities has been derived, and at 25° appears to hold true over a ten million-fold variation in catalyst (hydroxyl ion) concentration. The activation energies and frequency factors for both the hydrolyses of protonated naphazoline and unprotonated naphazoline have been experimentally determined. The reaction involving protonated naphazoline is favored by frequency while the reaction involving unprotonated naphazoline is favored by energy. The frequency effect is the greater so that, at normal temperatures, protonated naphazoline hydrolyzes about one thousand times faster than the unprotonated form. Postulations for the mechanisms of the reactions have been made in light of the kinetic data and a generalized equation for calculating the observed rate constant has been devised.**

ALTHOUGH the hydrolytic degradation of esters, amides, imides, and other carbonyl compounds is well recognized, the ability of other structures to degrade through a hydrolytic mechanism is often overlooked. Such oversights may be particularly costly in pharmaceutical research. Excellent examples of such "other" species are the imidazolines. It is, therefore, the purpose of the present communication to describe the kinetics of the specific base-catalyzed hydrolysis of a typical imidazoline—naphazoline.

Naphazoline, 2-(1-naphthylmethyl)-2-imidazoline, has been shown to be relatively stable in acidic or neutral solutions but readily prone to hydrolysis in basic solutions. The first step in this reaction (1) results in the formation of 1-naphthylacetylethylenediamine, which, upon more vigorous treatment (2), undergoes further cleavage to form 1-naphthylacetic acid and ethylenediamine. These reactions can be represented by the following scheme:

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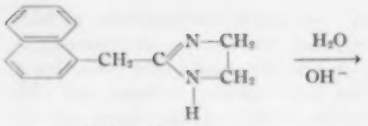
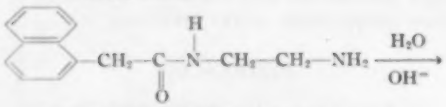
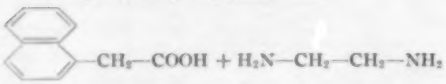
† American Foundation for Pharmaceutical Education Fellow 1956–1957.
‡ Present address: Merck Sharp and Dohme Research Laboratories, West Point, Pa.
This paper is based on a thesis presented to The Graduate School of Rutgers University by Marvin J. Stern in partial fulfillment of the requirements for the degree of Master of Science.
Portions of this paper are adapted from the honorable mention-winning manuscript submitted by Marvin J. Stern in the Eastern Region, 1958, Lunsford Richardson Pharmacy Award competition.
The authors wish to thank Mr. Jack Cooper of Ciba Pharmaceutical Products, Inc., for his generosity in supplying them with the naphazoline hydrochloride.



Naphazoline



1-Naphthylacetylethylenediamine



1-Naphthylacetic Acid Ethylenediamine

While both steps in the foregoing reaction scheme offer interesting subjects for kinetic studies, it is obvious that only the first step would be of concern in pharmaceutical formulation efforts, since this step is responsible for the disappearance of the intact physiologically active species from its solutions. As a result, it was decided to examine the kinetics of this reaction in detail with the further objective of utilizing the kinetic data in proposing a reaction mechanism.

## THEORETICAL CONSIDERATIONS

Naphazoline, like all weak bases, exists in aqueous solutions in both protonated (salt) and unprotonated (base) forms. As a result, any equation describing the rate of hydrolysis must include both potential substrates. The rate expression would therefore be given by

$$-d[\text{Naph}]_T/dt = k_{\text{OH}^-,\,S}\,[\text{OH}^-]^m[\text{Naph}]_S^{n} + k_{\text{OH}^-,\,B}\,[\text{OH}^-]^{m'}[\text{Naph}]_B^{n'} \quad \text{(Eq. 1)}$$

where the subscripts $S$, $B$, and $T$ refer to the salt, base, and total, respectively, and the superscripts are the orders of the reactions with respect to the different species. At constant hydroxyl ion activity, this becomes

$$-d[\text{Naph}]_T/dt = k_S'[\text{Naph}]_S^{n} + k_B'[\text{Naph}]_B^{n'} \quad \text{(Eq. 2)}$$

where $k_S'$ and $k_B'$ are the pseudo-$n$th and pseudo-$n'$th order rate constants for the salt and base forms, respectively. Dividing through by $[\text{Naph}]_T$ gives

$$-\frac{d[\text{Naph}]_T}{[\text{Naph}]_T} = \left(k_S'\frac{[\text{Naph}]_S^{n}}{[\text{Naph}]_T} + k_B'\frac{[\text{Naph}]_B^{n'}}{[\text{Naph}]_T}\right)dt \quad \text{(Eq. 3)}$$

If it is assumed that $n$ and $n'$ are both equal to unity (i.e., the reaction is first order with respect to either form of naphazoline), the terms $[\text{Naph}]_S/[\text{Naph}]_T$ and $[\text{Naph}]_B/[\text{Naph}]_T$ will be constant under conditions of constant pH. Integration of Eq. 3 will, therefore, give

$$\log [\text{Naph}]_T = -\frac{(k_S'[\text{Naph}]_S + k_B'[\text{Naph}]_B)t}{2.30\,[\text{Naph}]_T} + \frac{\log [\text{Naph}]_{T,o}}{2.30} \quad \text{(Eq. 4)}$$

where $[\text{Naph}]_{T,o}$ is the initial total naphazoline concentration. The overall reaction would, therefore, follow pseudo-first order kinetics with the specific rate constant ($k_{obs}'$) given by the equation

$$k_{obs}' = \frac{k_S'[\text{Naph}]_S + k_B'[\text{Naph}]_B}{[\text{Naph}]_T} \quad \text{(Eq. 5)}$$

In other words, the observed specific pseudo-first order rate constant is the "weighted average" of the individual salt and base constants.

If the reaction involving the protonated form and the one involving the unprotonated form are of identical order with respect to hydroxyl ion, the relationship

$$k_{\text{OH}^-,\,T} = \frac{k_{\text{OH}^-,\,S}[\text{Naph}]_S + k_{\text{OH}^-,\,B}[\text{Naph}]_B}{[\text{Naph}]_T} \quad \text{(Eq. 6)}$$

can also be written. In solutions of constant hydroxyl ion activity the expression relating the observed rate constant to the true rate constant is given by

$$k_{obs}' = k_{\text{OH}^-,\,T}[\text{OH}^-]^m \quad \text{(Eq. 7)}$$

Substitution of Eq. 6 in Eq. 7 gives, upon taking logarithms

$$\log k_{obs}' = -m\text{pOH} + \log \frac{k_{\text{OH}^-,\,S}[\text{Naph}]_S + k_{\text{OH}^-,\,B}[\text{Naph}]_B}{[\text{Naph}]_T} \quad \text{(Eq. 8)}$$

The relative concentrations of the two forms of naphazoline can be determined by the buffer equation which in this case is

$$\log ([\text{Naph}]_S/[\text{Naph}]_B) = \text{p}K_a - \text{p}H \quad \text{(Eq. 9)}$$

where $K_a$ is the dissociation constant of the protonated form, the conjugate acid of naphazoline base. Solving Eq. 9 for $[\text{Naph}]_S$ and $[\text{Naph}]_B$ in terms of $[\text{Naph}]_T$ gives

$$[\text{Naph}]_S = \frac{[\text{Naph}]_T[\text{H}^+]}{K_a + [\text{H}^+]} \quad \text{(Eq. 10)}$$

and

$$[\text{Naph}]_B = \frac{[\text{Naph}]_T K_a}{K_a + [\text{H}^+]} \quad \text{(Eq. 11)}$$

Substituting these values in Eq. 8 gives

$$\log k_{obs}' = -m\text{pOH} + \log\left(\frac{k_{\text{OH}^-,\,S}\,[\text{H}^+] + k_{\text{OH}^-,\,B}\,K_a}{K_a + [\text{H}^+]}\right) \quad \text{(Eq. 12)}$$

In solutions of low hydroxyl ion activity naphazoline exists almost entirely in the protonated form. Equation 8 therefore becomes

$$\log k_{obs}' = -m\text{pOH} + \log k_{\text{OH}^-,\,S} \quad \text{(Eq. 13)}$$

Since $k_{\text{OH}^-,\,S}$ is constant at any particular temperature, if Eq. 13 holds, a plot of log $k_{obs}'$ *versus* pOH will be linear. The slope of the line will be equal to the negative of the order of the reaction with respect to hydroxyl ion. The "$y$" intercept of any such line will, of course, be equal to log $k_{\text{OH}^-,\,S}$.

It will be shown in the following sections that the above equations appear to hold true and the assumptions made in their derivations are valid.

## EXPERIMENTAL

**Determination of Dissociation Constants.**—The dissociation constant of naphazoline conjugate acid at each of three temperatures was determined by carefully preparing volumetric solutions of known concentrations of naphazoline hydrochloride and free naphazoline base.[1] This was accomplished by dissolving a known weight of the salt in recently boiled, double-distilled water and adding a known volume of a standard sodium hydroxide solution. Enough sodium chloride to bring the ionic strength to 0.6 was added and the solution was brought to volume with recently boiled, double-distilled water. The pH of the solution was immediately measured at the desired temperature using a Beckman Model H2 pH meter. The pKa value was calculated from Eq. 9.

The values of the dissociation constants so obtained were "semi-classical" values, i.e., based on hydrogen ion activities and all other values as concentrations. Since all of the measurements and calculations involved are actually in these units, the "semiclassical" dissociation constants are the proper values to use. Due to the presence of concentration terms, these constants had to be measured under conditions of the same ionic sternth as the experimental naphazoline solutions in order to be meaningful.

**Buffer Systems.**—Buffer solutions were utilized in order to keep the hydroxyl ion activities constant during the kinetic runs. To obviate any error due to the effect of ionic strength on the reaction rates, all buffers were made to have ionic strengths of exactly 0.6 by adjustment with sodium chloride. The buffers were prepared by blending a solution of the base (or acid) at $\mu = 0.6$ and a solution of its salt at $\mu = 0.6$ until the desired pH was obtained. The solutions were made 0.2 *M* with respect to total buffer constitutents wherever the solubility permitted. The ionic strength figure of 0.6 was chosen since this represents the ionic strength of a 0.2 *M* solution of a 1:2 or 2:1 electrolyte.

Buffers were prepared to cover the pH range of approximately 6 to 14. The buffer types, in order of increasing pH, were: triethanolamine-triethanolamine hydrochloride, sodium borate-boric acid, sodium carbonate-sodium bicarbonate, diethylamine-diethylamine hydrochloride, and for very high pH's (above 12), dilute solutions of sodium hydroxide were used. The usual calcium or barium hydroxide could not be used due to the presence of sodium bicarbonate in the assay procedure (3). Phosphate salts also could not be used in buffers for this work since their presence seemed to prevent the colored complex developed in the assay from adhering to Beer's law, even in very dilute solutions.

The highest pH obtainable was 13.48 at 25°, corresponding to a 0.6 *M* sodium hydroxide solution. The fact that the buffer solutions were all made to have ionic strengths of 0.6 precluded the use of more concentrated hydroxide solutions.

[1] Due to the low solubility of the naphazoline base, the solutions were made on the order of $2.5 \times 10^{-3}$ *M* with respect to the salt and base.

An important consideration in the use of buffer solutions is the fact that the hydroxyl ion activity changes with temperature by virtue of the thermal change of the value of the ion product of water. The change in hydroxyl ion activity is especially evident in the case of acid-salt buffers where the hydronium ion activity is kept relatively constant and changes in the ion product of water are reflected, for the most part, in changes in the activity of hydroxyl ion. Even in base-salt buffers, however, there is an appreciable thermal change in hydroxyl ion activity. The common practice of ignoring these changes was considered to be highly inaccurate, since calculations show that in a reaction first order with respect to hydroxyl ion, a change of as little as 0.2 pOH units represents an error of about 60% in the specific rate constant. This error is even greater in reactions of higher order with respect to hydroxyl ion.

In order to determine the correct values of the hydroxyl ion activities, the pH of each buffer was measured at the temperatures at which the kinetic runs were made. The pH's were subtracted from the thermodynamic pKw's at the temperatures in question (4) to give the pOH's.

**Kinetic Runs.**—One hundred milligrams of naphazoline hydrochloride was placed in a 100-ml. glass-stoppered volumetric flask, enough buffer solution added to make 100 ml., and the solution was filtered. The flask was placed in a constant temperature oil bath. After thermal equilibrium had been attained, a 5-ml. sample was removed from the flask, delivered into a glass vial, stoppered, and plunged into an ice-water mixture. In the case of high pH systems, where sudden cooling was not sufficient to quench the reaction, the vial contained a predetermined amount of hydrochloric acid sufficient to give a final pH of about 6. This sample was arbitrarily designated as the "zero-hour" sample. Additional samples were removed and quenched after appropriate time intervals. All samples were refrigerated until the time of assay.

Routine analyses for residual naphazoline were carried out by the colorimetric method of Slack and Mader (3). Prior to color development, the solutions were diluted with sufficient buffer so that the absorbance of the colored complex did not exceed 0.4. At higher absorbances, the buffer constituents appear to cause deviations from Beer's law.

The analytical procedure used to verify the stoichiometry of the reactions was the partition chromatographic procedure of Schwartz, Kuramoto, and Malspeis (2), as modified by Stern (5).

All volumetric withdrawals from the refrigerated samples were done with the vials at 0°.

## RESULTS AND DISCUSSION

**Dissociation Constants.**—The pKa values of naphazoline conjugate acid were determined as described in the experimental section. Three solutions of different base:salt ratios were used for the determination of each pKa value and the average was taken. These "semiclassical" pKa values at 0.6 inonic strength are given in Table I. These values are in very good agreement with the results of Hall and Sprinkle (6) who found that the pKa's of the conjugate acids of bases of about the strength of

TABLE I.—SEMICLASSICAL DISSOCIATION CONSTANTS OF NAPHAZOLINE CONJUGATE ACID AT $\mu = 0.6$

| Temperature, °C. | pKa |
|---|---|
| 25.0 | 10.35 ± 0.02 |
| 35.0 | 10.13 ± 0.02 |
| 45.0 | 9.92 ± 0.03 |

naphazoline showed a linear decrease of about 0.02 for each degree increase in temperature.

**Verification of the Stoichiometry of the Reaction.**—In order to show that hydrolysis was the only degradative pathway under the conditions employed in the kinetic runs, it was necessary to verify the stoichiometry of the reaction. A kinetic run in pOH 4.55 borate buffer at 65° was chosen for this since this pOH and temperature were considered to be most representative of the conditions employed in the subsequent kinetic runs. This buffer also corresponded in pH to the internal phase of the Celite 545 partition column employed in the assay (2, 5).

The samples were assayed for naphazoline, 1-naphthylacetylethylenediamine, and 1-naphthylacetic acid. The results are illustrated graphically in Fig. 1. This plot clearly shows that hydrolysis is the only significant degradative pathway under the conditions employed since all of the naphazoline was accounted for and the total molar concentration of naphazoline plus its hydrolytic degradation products was constant. The 1-naphthylacetylethylenediamine did not hydrolyze to any significant extent to form 1-naphthylacetic acid.

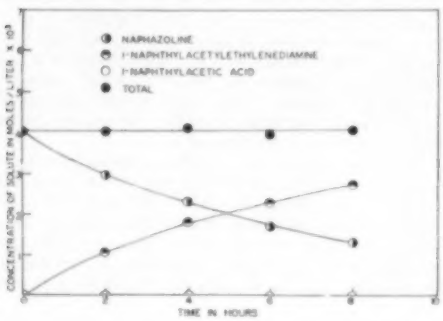



Fig. 1.—Verification of the stoichiometry of the reactions.

**Effect of Naphazoline Concentration.**—In Fig. 2, the residual naphazoline concentrations from the kinetic run in pOH 4.55 buffer at 65° are plotted logarithmically against time. From the straight line obtained, it appears that the rate of hydrolysis exhibits a first order dependency on the naphazoline concentration. The fact that the same line was obtained when calculated from direct analysis of the residual naphazoline or from the analyzed concentrations of the decomposition products further illustrates the stoichiometry of the degradation. All of the subsequent kinetic runs throughout the entire temperature and pH ranges that were investigated showed similar first order dependencies. From the theoretical considerations presented, this would certainly indicate that the reaction is first order with respect to naphazoline regardless of whether the imidazoline exists as the protonated or unprotonated species.

**Effect of Hydroxyl Ion Activity.**—In Fig. 3 the logarithms of the pseudo-first order rate constants have been plotted against pOH for the range where naphazoline exists almost exclusively in the protonated form. Straight lines with slopes of −1.00 have been obtained, which, from Eq. 13, indicates that the rate of hydrolysis of naphazoline is first order with respect to hydroxyl ion. The plots have

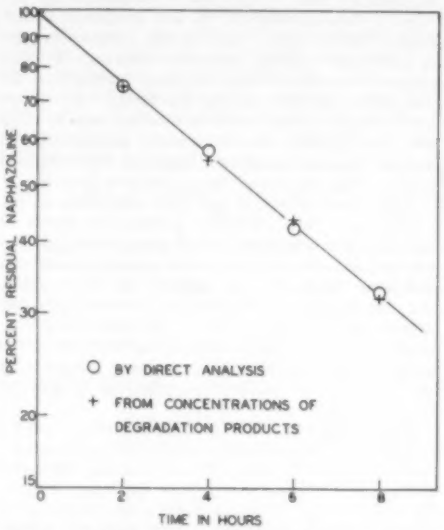



Fig. 2.—Kinetic run at 65.0° in 0.1 *M* borate buffer; $\mu = 0.6$; pOH = 4.55.

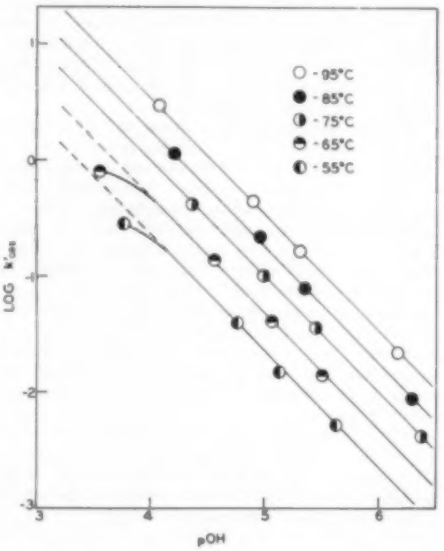



Fig. 3.—Effect of hydroxyl ion activity on the rate of hydrolysis.

been extrapolated to the "$y$" intercepts to obtain the logarithms of the second order specific rate constants which appear in Table II.

It can be seen in Fig. 3 that the points in the vicinity of pOH 3.5–3.8 do not fall on the lines, but below them. These deviations are in accord with the theoretical considerations governing high pH (low pOH) systems where the presence of the unprotonated form of naphazoline is greatly increased. All other things being equal, the unprotonated base would react at a slower rate than the protonated salt form due to the coulombic attraction between the latter and hydroxyl ions.

The effect of hydroxyl ion on the rate of hydrolysis at 25° in the pOH range 0–8 is given in Fig. 4. While the points were experimentally determined, the line was not fitted to the experimental data, but instead, was drawn to represent the relationship expressed by Eq. 12. The values of $k_{OH^-, B}$ and $k_{OH^-, B}$ used in solving Eq. 12 were 182 liters moles$^{-1}$ hours$^{-1}$ and 0.187 liters moles$^{-1}$ hours$^{-1}$, respectively. The value of $k_{OH^-, B}$ was obtained by extrapolation from results at higher temperatures. The kinetic run at 25° at pH 12.98 was used for the determination of $k_{OH^-, B}$.

Inasmuch as the derivation of Eq. 12 required that the hydrolyses of both protonated and unprotonated naphazoline have the same kinetic dependency on hydroxyl ion, the excellent agreement shown in Fig. 4 between the experimental results and theoretical equation indicates that this requirement was met. Since it has been shown that the hydrolysis of protonated naphazoline is first order with respect to hydroxyl ion, the hydrolysis of unprotonated naphazoline must also be first order with respect to this ion.

**Temperature Dependencies of the Reactions.**—The Arrhenius-type plots which appear in Figs. 5 and 6 show the temperature dependencies of the reactions. Difficulty was encountered during attempts to determine the effect of temperature on the rate of hydrolysis of the unprotonated form of naphazoline. The difficulty evolved from the fact that at normal temperatures the specific rate of hydrolysis of the protonated form is about one thousand times faster than that of the unpro-

TABLE II.—SPECIFIC RATE CONSTANTS AT ELEVATED TEMPERATURES FOR THE HYDROLYSIS OF PROTONATED NAPHAZOLINE

| Temperature °C. | $k_{OH^-, S}$, liters moles$^{-1}$ hours$^{-1}$ |
|---|---|
| 95 | $3.4 \times 10^4$ |
| 85 | $1.8 \times 10^4$ |
| 75 | $9.8 \times 10^3$ |
| 65 | $4.7 \times 10^3$ |
| 55 | $2.2 \times 10^3$ |

The temperatures were controlled to ±0.05° or better.

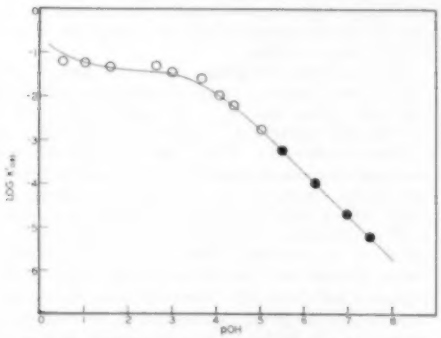



Fig. 4.—Effect of hydroxyl ion activity on the rate of hydrolysis at 25.0°. The line represents the relationship expressed by Eq. 12. The open circles are experimental points determined at 25.0°. The solid circles are experimental points determined at other temperatures and extrapolated to 25.0°.

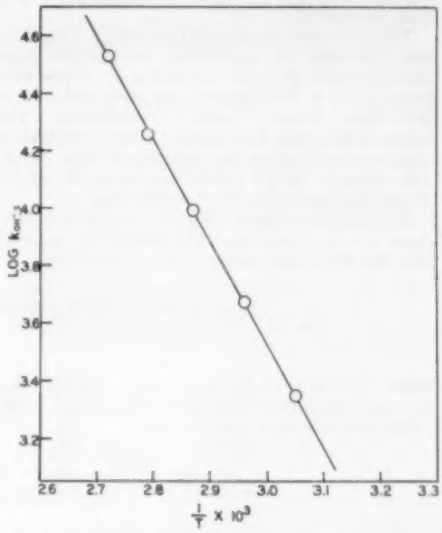



Fig. 5.—Effect of temperature on the rate of hydrolysis of protonated naphazoline.

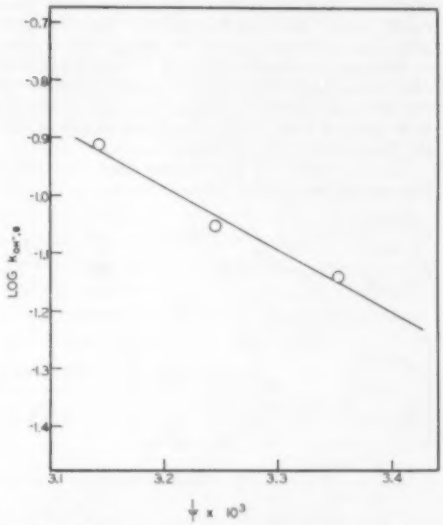



Fig. 6.—Effect of temperature on the rate of hydrolysis of unprotonated naphazoline.

tonated form. Thus, even at pH 13.48, the highest pH that could be used, where the concentration of the unprotonated form is over thirteen hundred times greater than the concentration of the protonated form, the hydrolysis of the latter still accounts for over two-fifths of the overall degradation rate. Any rate constant for the hydrolysis of the unprotonated form derived under such conditions is, at best, an approximation, as would be any thermodynamic or kinetic value calculated from such rate constants. It was felt, however, that such approximations would still yield useful information. In Fig. 6, the regression line was drawn using the method of least squares (7).

The activation energy and frequency factor for the hydrolysis of protonated naphazoline have been calculated from the plot in Fig. 5. These were found to be 16.4 kilocalories per mole and $1.91 \times 10^{14}$ liters moles$^{-1}$ hours$^{-1}$, respectively. The values of the same parameters for the hydrolysis of unprotonated naphazoline, determined from Fig. 6, were found to be 4.9 kilocalories per mole and $3.0 \times 10^{9}$ liters moles$^{-1}$ hours$^{-1}$, respectively.

A generalized equation for the specific rate constant for the base-catalyzed hydrolysis, modeled after the Arrhenius equation, can be written as

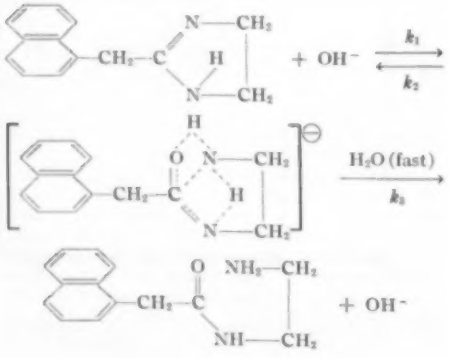
$$k_{obs}' = \frac{(1.91 \times 10^{14}\, e^{-16,400/1.99\tau}\,[\text{Naph}]_S + 3.0 \times 10^{9}\, e^{-4,900/1.99\tau}\,[\text{Naph}]_B)[\text{OH}^-]}{[\text{Naph}]_T} \quad \text{(Eq. 14)}$$

where $\tau$ is the absolute temperature. Substituting the equivalents previously derived for $[\text{Naph}]_S$ and $[\text{Naph}]_B$ (see Eqs. 10 and 11) gives

$$k_{obs}' = \frac{1.91 \times 10^{14}\, e^{-16,400/1.99\tau}\, K_w + 3.0 \times 10^{9}\, e^{-4,900/1.99\tau}\, K_a[\text{OH}^-]}{K_a + [\text{H}^+]} \quad \text{(Eq. 15)}$$

**Mechanisms of the Reactions.**—While the kinetic data which have been presented are valuable in applications such as stability evaluation, they have much more fundmental utility. Kinetic data offer one important means of testing the validity of reaction mechanisms, and it is with the knowledge of mechanisms that the ultimate in predictions can be made. For this reason, mechanisms for the hydrolysis of naphazoline, which are in accord with the kinetic data, are proposed.

The hydrolysis of naphazoline base can follow the course represented by



While the exact nature of the intermediate cannot be unambiguously proved, the depicted structure represents a hybrid or compilation of the resonant forms that can be postulated. A model of the intermediate, constructed from Godfrey Molecular Models (8), indicates that the proposed structure is feasible.

The second step in the reaction, replacement of the hydroxyl group of the water, would be very fast. In other words, almost as soon as the unstable intermediate is formed, it reacts with the water. The value of $k_3$, therefore, would be much larger than that of either $k_1$ or $k_2$. Applying the steady state treatment to these reactions gives the following rate equation:

$$-d[\text{Naph}]/dt = k_1\left(1 - \frac{k_2}{k_2 + k_3[\text{H}_2\text{O}]}\right)[\text{Naph}][\text{OH}^-] \quad \text{(Eq. 16)}$$

Since in dilute aqueous solutions, all of the terms within the parentheses are constant, they can be combined with $k_1$ to give an overall second order expression:

$$-d[\text{Naph}]/dt = k_{\text{OH}^-}[\text{Naph}][\text{OH}^-] \quad \text{(Eq. 17)}$$

The value of $k_{\text{OH}^-}$ should be very close to that of $k_1$ since the term $k_2/(k_2+k_3[H_2O])$ would be very small due to the large values of $k_2$ and $[H_2O]$.

According to Eq. 17, the reaction would be first order with respect to both naphazoline and hydroxyl ion. The experimental data appear to satisfy this requirement.

The fact that, in the proposed mechanism, hydroxyl ion is both a reactant and a product would make the reaction a true specific base-catalyzed hydrolysis.

Although the depicted mechanism is for the hydrolysis of unprotonated naphazoline, a very similar scheme can be proposed for the hydrolysis of protonated napthazoline.

The Arrhenius frequency factor of $1.91 \times 10^{14}$ liters moles$^{-1}$ hours$^{-1}$ for the reaction involving protonated naphazoline is a "normal" value for a bimolecular reaction in solution as calculated from collision theory (9). Upon cursory consideration, one might expect this parameter to be considerably greater by virtue of the fact that the reacting entities are of opposite electrical charge and, thus, exhibit coulombic attraction. This, however, is offset by two factors. First, in ionic interactions, the hydration shell has a restraining effect on the ions and will thus slow down the rate of collision. Second, the proposed mechanism of the reaction is such that the hydroxyl ion can attack from only one side of the naphazoline molecule, *viz.*, the side in line with the double-bonded imino nitrogen atom. For this reason, not every collision between ions of the proper energy will result in a reaction and the frequency factor will be lowered. The much lower

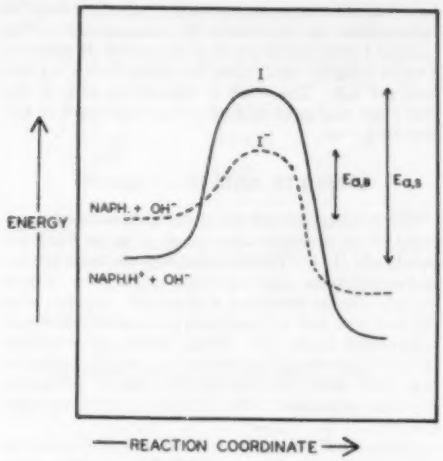



Fig. 7.—Energetics of the reactions.

frequency factor ($3.0 \times 10^9$ liters moles$^{-1}$ hours$^{-1}$) for the reaction involving unprotonated naphazoline is also to be expected since the frequency of collision for an ion and a neutral molecule would be much lower than for two oppositely charged ions.

The relative activation energies can best be explained by the energy diagram in Fig. 7. Naphazoline conjugate acid, being charged, is more stable in solution than is the uncharged naphazoline base. Similarly, the activated complex formed in the reaction involving the free base has a charge and is more stable in solution than the uncharged intermediate in the reaction involving the conjugate acid (protonated) form. Since the activation energy is the difference in energy between the reactants and the activated complex, this parameter for the free base reaction would be considerably less than for the conjugate acid reaction. The experimental values of 4.9 kilocalories per mole and 16.4 kilocalories per mole, respectively, agree with the above considerations.

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# Analysis of Adrenergic Drug Action*

By R. S. McCUTCHEON† and RAYMOND P. AHLQUIST

**Three analogs of arterenol were comparatively studied on several effector systems in dogs. The amines were: (*a*) ethylnorepinephrine (butanephrine), (*b*) isoproterenol (Isuprel), and (*c*) the N-isopropyl derivative of ethylnorepinephrine (Win-3046). These amines were compared to levarterenol and epinephrine as to potency and effect on arterial pressure, heart rate, intestinal motility, splenic capsule, and urine output. The effects on the splenic capsule best illustrates the general relationships found. Epinephrine, levarterenol, and ethylnorepinephrine produce primary splenic contraction; this effect is prevented by adrenergic blockade and is not modified by ganglionic blockade. Compounds (*b*) and (*c*), and (*a*) to some extent, induce a reflex splenic contraction by their depressor action. This effect is prevented by either adrenergic or ganglionic blockade. When administered simultaneously with epinephrine, (*b*) and (*c*) diminish the splenic contraction produced by the epinephrine.**

THE ADRENERGIC RECEPTOR is the primary site of action of the adrenergic neurohormone and adrenergic drugs. It has been proposed that the adrenergic receptor occurs in two different forms (1). As far as smooth muscle is concerned, one of these receptors, known as the *alpha* receptor serves primarily excitatory responses. The other receptor, known as the *beta* receptor, subserves primarily inhibitory responses. Some adrenergic drugs activate both receptors. Therefore, it would be desirable to have a convenient method for determining the differential potency on these two receptors. To investigate such a method, a comparative study in the anesthetized dog has been carried out using epinephrine, levarterenol, isoproterenol, ethylnorepinephrine, and the N-isopropyl derivative of ethylnorepinephrine (Win-3046).

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† Experimental work done during tenure in Cardiovascular Research and Training Program, supported by Grant HTS-5044 from the National Heart Institute, Public Health Service. Present address: Oregon State College, School of Pharmacy, Corvallis.

## EXPERIMENTAL

**Method.**—Mongrel dogs of either sex weighing 10 to 15 Kg. were anesthetized with pentobarbital sodium, 10 to 15 mg./Kg., administered intravaneously thirty minutes after a subcutaneous injection of morphine sulfate, 10 mg./Kg. The arterial pressure was recorded from either the femoral or carotid artery by means of a mercury manometer or a Statham transducer. The contraction of the

splenic capsule was recorded by a method previously described by Ahlquist, *et al.* (2). In brief, this method consists of exposing the spleen through a midline incision, fixing one edge of the spleen to a rigid plastic support, and connecting the opposite edge to an isotonic displacement gauge. The amount of splenic contraction is recorded as centimeters of galvanometer trace deflection on the photokymographic record.

A series of six experiments were made on the gut, urine flow, and spleen. The drugs were injected alternately in the following doses. Levarterenol, epinephrine, and isoproterenol were each given in doses of 0.01 ml./Kg. of a 0.001 *M* solution. Win-3046 was injected in a dose of 0.02 ml./Kg. of a 0.001 *M* solution, and ethylnorepinephrine as 0.02 ml./Kg. of 0.01 *M* solution. These doses were, at times, doubled or halved in order to observe the relative responses better. In every case, in this and subsequent injections, decomposition of the amines was prevented by dissolving them in a solution of chlorobutanol and sodium bisulfite, 0.1% each. When blocking drugs were used they were injected in the doses shown below. The number of injections per experiment varied from 20 to 30. Control levels were obtained for each of the responses being measured and comparisons were made with the responses produced with the drugs under investigation, both before and after the use of the blocking agents. After each injection the response was allowed to return to control levels before the next injection. In these experiments the urine output from the renal pelvis was recorded with an electronic drop counter through a catheter inserted into the ureter. The activity of the small intestine of the animals in the same series was recorded by means of a small, water-filled balloon inserted into the lumen of the gut. The pressure developed by each intestinal contraction was recorded by means of a Statham transducer.

In another series of 15 experiments, dogs were given a constant infusion of one of the following drugs: ethylnorepinephrine, Win-3046, levarterenol, or isoproterenol and during this infusion each of the other drugs was injected intravenously every five minutes until each had been injected five to eight times. The infusions were administered by means of a drip bottle or by a syringe actuated by a stepping relay controlled through an electronic counting device (3). Ethylnorepinephrine was infused at a rate of 21.5 mcg./Kg./minute, Win-3046 was infused at a rate of 25.3 mcg./Kg./minute, levarterenol at a rate of 3.13 mcg./Kg./minute, and isoproterenol at a rate of 2.28 mcg./Kg./minute. In each case the infusions lasted one hundred to one hundred and twenty minutes. These doses were adjusted to give comparable results in each case. A few of the animals were given atropine, 0.5 mg./Kg., and when appropriate, mecamylamine (Inversine), 1 mg./Kg., was used as a ganglionic blocking agent or phenoxybenzamine (Dibenzyline) was used as an adrenergic blocking agent in a dose of 5 mg./Kg. All drugs were injected intravenously. A comparison was made of the results of the injections with the controls and with the results obtained after each type of block. During the infusions, blood pressure was recorded as indicated above and heart rates were recorded with an electrocardiograph and counted from the photokymographic tracing.

In comparative studies on adrenergic drugs we have found it convenient to use a "standard dose" of epinephrine as the basis for comparison. This standard dose is 0.01 ml./Kg. of a 0.001 *M* solution. This is roughly equivalent to about 2 mcg. of free base per Kg. The doses of the amines used in this study are expressed as fractions or multiples of this standard dose.

## RESULTS AND DISCUSSION

The comparative effects of the amines on mean arterial blood pressure were found to be as described previously (1, 4). Levarterenol was the most potent, and epinephrine was somewhat less active. Ethylnorepinephrine produced a depressor response with the first dose and an increasing pressor response with subsequent doses (5). When given by infusion, ethylnorepinephrine produced a pressor response. Win-3046 and isoproterenol produced consistent depressor responses with the latter being the more active (6).

Following Dibenzyline, levarterenol produced only a small pressor response while all four of the other amines produced only depressor responses. These results indicate that levarterenol affects primarily the *alpha* receptor, epinephrine and ethylnorepinephrine affect both the *alpha* and *beta* receptors, while Win-3046 and isoproterenol affect only the *beta* receptor. When combinations of the drugs were injected, the results were not conclusive.

As has been previously described (1) acute changes in urine output roughly shows the relative renal vasoconstricting action of substances such as epinephrine. However, only with epinephrine and levarterenol was any consistent result found. With the other amines the urine output varied with the arterial pressure and no conclusions could be drawn regarding the relative action of these substances on the renal vascular bed.

The intestine of dogs not receiving atropine was relaxed by all of the amines except isoproterenol. This amine usually produced intestinal contraction. Following atropine, however, isoproterenol produced only relaxation. The relative potencies were as follows: isoproterenol was equal or more potent than epinephrine with the others less potent in this order: levarterenol, ethylnorepinephrine, and Win-3046. Mecamylamine did not affect these responses.

These results fit fairly well with the concept that the adrenergic inhibitor receptor of the gut is of the *alpha* type (2). The fact that isoproterenol is often more potent in this regard than epinephrine, however, is not consistent with this view. Recent studies have indicated that the canine ileum has both *alpha* and *beta* receptors, and that both receptors subserve relaxation (7, 8). We can conclude that the effect of these amines on the intact intestine is not a good criterion for relative receptor potency.

As previously reported (1), epinephrine was found to be the most potent substance in producing contractions of the splenic capsule. All of the other amines studied also produced splenic contraction when injected intravenously. The mechanism of action, however, was not the same for each. Figure 1 illustrates the control responses to epinephrine, isoproterenol, and Win-3046. As shown in Figs. 2 and 3, following the administration of a ganglionic blocking agent the splenic contraction produced by isoproterenol is abolished. The effect of Win-3046 is

TABLE I.—AVERAGE SPLENIC CONTRACTION PRODUCED BY THE DRUGS SHOWN[a]

| Dose | Epinephrine | Levarterenol | Butanephrine | Win-3046 | Isoproterenol |
|---|---|---|---|---|---|
| S. D.[b] | 5.91 | 1.92 | (×10) 0.32 | (×20) 2.37 | 0.81 |
| $\frac{\text{S. D.}}{2}$ | 3.83 | ... | ... | ... | ... |
| S. D. PM[c] | 6.15 | 2.91 | 1.57 | ... | ... |
| $\frac{\text{S. D. PM}}{2}$ | 4.22 | ... | ... | ... | ... |

[a] Deflection on photokymographic record. Contractions in centimeters, obtained from six animals with 18 to 30 injections per animal.
[b] S. D. refers to standard dose.
[c] PM is post-mecamylamine (Inversine).

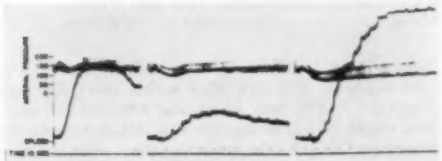


Fig. 1.—Control responses of splenic contractions to epinephrine, isoproterenol, and Win-3046.

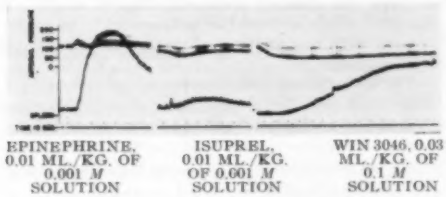


Fig. 2.—Splenic contractions after administration of Inversine, 1 mg./Kg.

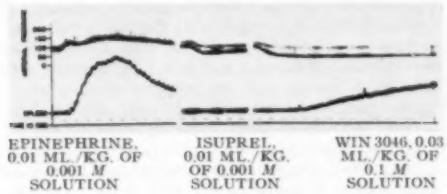


Fig. 3.—Splenic contractions after second dose of Inversine, 1 mg./Kg.

markedly reduced but this amine continues to produce some splenic contraction.

Levarterenol and ethylnorepinephrine both produce splenic contraction. These substances are potentiated by ganglionic blockade. As described by Cameron (5) for the pressor response, ethylnorepinephrine becomes increasingly more potent in producing splenic contraction when repeated doses are given.

These results indicate that the amines can produce contraction of the splenic capsule both by direct action and by reflex action due to the fall in blood pressure. The latter effect is prevented by ganglionic blockade.

Assuming then that splenic smooth muscle has only *alpha* receptors, results indicate that epinephrine is the most potent *alpha* activator, followed by levarterenol, ethylnorepinephrine, and Win-3046. Isoproterenol, having practically no action on *alpha* receptors, produces splenic contractions only through reflex action. The results can be seen in Table I.

However, some evidence has been obtained to show that the splenic capsule has some adrenergic *beta* receptors. This is shown by administering epinephrine and Win-3046 simultaneously. In every case the spleen contracted less when the mixture was given than when the same amount of epinephrine was given alone. If both substances were producing splenic contraction by the same mechanism the effect should be additive and a greater contraction should result. Since this is not the case one must assume that both epinephrine and Win-3046 activate some *beta* receptors in the spleen. When the drugs are acting together the sum of this *beta* action is greater than the sum of the *alpha* effect of epinephrine and Win-3046. At the same time the epinephrine in the mixture prevents the depressor response to Win-3046 so that the reflex effect of the latter in causing contraction of the spleen is largely lost. Therefore, the adrenergic inhibitory action on the spleen becomes more apparent.

Table II shows the relative adrenergic receptor-activating potencies of the five catecholamines based primarily on their effects on splenic contraction.

TABLE II.—RELATIVE ADRENERGIC RECEPTOR-ACTIVATING POTENCIES

| | alpha | beta |
|---|---|---|
| Epinephrine | +++++ | ++++ |
| Levarterenol | ++++ | + |
| Ethylnorepinephrine | ++ | ++ |
| Win-3046 | + | +++ |
| Isoproterenol | + | +++++ |

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# Activation Analysis of Certain Arsenic and Antimony-Containing Pharmaceuticals and the Effect of Beta-Emitting Isotopes*

By DAVID G. KAISER† and JOHN E. CHRISTIAN

**Slow neutron activation analysis has been applied successfully to the analysis of pure samples of arsenic trioxide, carbarsone, oxophenarsine, antimony potassium tartrate, and stibophen, the average error being between 4 and 5 per cent. Sample processing and chemical separations were not required in the procedures. The effect of beta-emitting isotopes on the gamma-ray scintillation spectrometer was investigated and the detection levels of phosphorus-32 determined with and without shielding.**

PERHAPS the earliest examples of neutron activation analysis were reported by Hevesy and and Levi (1, 2, 3) who applied the procedure to the investigation of rare earth elements. The determination of arsenic in germanium dioxide was described by Smales and Brown (4) while Griffon and Barband (5) studied the distribution of arsenic in hair. Smales and Pate (6, 7, 8) published a series of papers on the determination of submicrogram quantities of arsenic. Hudgens and Cali (9) utilized the method to analyze for antimony in zirconium oxide. An analysis for arsenic in silicon was described by James and Richards (10). Recently, Smales, Mapper, Wood, and Salmon (11) determined trace quantities of arsenic, antimony, and copper in pure silicon.

The work reported here consisted of investigating the slow neutron activation analysis of arsenic and antimony-containing pharmaceuticals.

## EXPERIMENTAL

**Analysis of Arsenic and Antimony-Containing Pharmaceuticals.**—For these experiments, polyethylene tubing (2 inches long × 3/32 inch internal diameter × 1/4 inch external diameter) was utilized as a sample container. The individual pharmaceuticals were dried according to the U. S. P. XV or N. F. X directions, and a sample placed in a 2-inch length of the tubing. The heat-sealed polyethylene tubes were then placed in an aluminum spacer which was subsequently inserted into a standard ANL[1] aluminum irradiatio ncontainer (6 inches high × 1 1/4 inches diameter). Samples prepared in this manner were subjected to ~ 1 × $10^{8}$ neutrons per square cm. per second for twenty-four to forty-eight hours. The induced radioactivity was determined with a RIDL[2] scintillation spectrometer consisting of a model 115 single channel pulse height analyzer, model 200T scaler, model 43A scintillation counter with a thallium-activated sodium iodide well-type crystal (1 3/4 inch diameter × 2 inch thick with a well 0.650 inch diameter × 1 1/2 inch deep), and a model L-9 lead-iron shield. In later studies, a model 39-1 electronic sweep and count rate meter together with a Leeds and Northrup[3] Speedomax type G recorder were installed to provide an automatic recording instrument. The instrument was calibrated with cesium-137 and cobalt-60[4] to provide a maximum channel level of 1 Mev.[5] and a maximum channel width of 0.1 Mev. at a gain setting of 1/4. A gain of 1/8 doubled the channel level and channel width. A linearity study showed the instrument to be standardized with respect to peak energy *versus* channel level readings.

The investigated compounds and the selected solvents were as follows: arsenic trioxide and sodium hydroxide; carbarsone[6] and sodium hydroxide; oxophenarsine hydrochloride[7] and distilled water; antimony potassium tartrate and aqua regia; stibophen[8] and distilled water.

After irradiation, the polyethylene tubes were opened with a razor blade and the contents were transferred to tared, volumetric flasks. These samples were brought up to volume with the selected solvent and allowed to equilibrate thermally before pipetting an aliquot for analysis. One 500-λ portion was transferred into a 2 inch test tube together with two 500-λ rinsings and the test tube was stoppered with a paraffin-coated cork. In later studies, Neutraglas[9] ampuls of 5-ml. capacity were chosen for counting larger samples in the well-type crystal. The qualitative spectrum was determined by placing each sample in the counting well of the previously standardized gamma-ray scintillation spectrometer. Differential spectral determinations were performed at gains 1/4 and 1/8 with a channel width of "1." In each instance the spectrum compared with literature values and had the same energy distributions as standard reactor irradiated units.[10] Quantitative studies were delayed for twenty-four to forty-eight

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[1] ANL, Argonne National Laboratory.

[2] Radiation Instrument Development Laboratory, Chicago, Ill.

[3] Leeds and Northrup Co., Philadelphia, Pa.

[4] Cesium-137 and Cobalt-60 were obtained from the Oak Ridge National Laboratory, Oak Ridge, Tenn.

[5] Mev.—million electron volts.

[6] Supplied through the courtesy of Eli Lilly and Co., Indianapolis, Ind.

[7] Mapharsen, supplied through courtesy of Parke Davis and Co., Detroit, Mich.

[8] Fuadin, supplied through courtesy of Winthrop Laboratories, New York, N. Y.

[9] Neutraglas ampuls, product of Kimble Glass Co., Toledo, Ohio.

[10] Standard reactor irradiated units of arsenic-76 and antimony-122 were obtained from Oak Ridge National Laboratory, Oak Ridge, Tenn.

TABLE I.—RESULTS OF THE SLOW NEUTRON ACTIVATION ANALYSIS OF ARSENIC AND ANTIMONY-CONTAINING PHARMACEUTICALS[a]

| Compound Investigated | Sample Weight, Gm. | Total Activity, Counts/min. | Calculated Activity of 50-mg. Sample[b] Counts/min. | Per Cent Error[c] |
|---|---|---|---|---|
| Arsenic trioxide[d] | 0.1134 | 12,469 | 5,498 | 4.9 |
| | 0.2755 | 24,591 | 4,663 | |
| | 0.5405 | 49,120 | 4,544 | |
| | 0.5444 | 48,495 | 4,454 | |
| Carbarsone | 0.0530 | 61,304 | 57,835 | 0.3 |
| | 0.0819 | 94,169 | 57,487 | |
| | 0.1280 | 148,060 | 57,835 | |
| | 0.1512 | 176,887 | 58,492 | |
| Oxophenarsine | 0.1185 | 198,398 | 83,712 | 1.2 |
| | 0.1520 | 269,774 | 88,741 | |
| | 0.2444 | 427,564 | 87,472 | |
| | 0.2524 | 434,352 | 86,045 | |
| Antimony potassium tartrate[e] | 0.1396 | 4,618,418 | 1,654,161 | 6.1 |
| | 0.2325 | 9,436,490 | 2,029,352 | |
| | 0.3029 | 11,675,860 | 1,927,345 | |
| | 0.3742 | 11,701,100 | 1,563,482 | |
| Stibophen | 0.0557 | 107,651 | 96,808 | 5.8 |
| | 0.1034 | 210,884 | 127,345 | |
| | 0.1667 | 388,255 | 116,453 | |
| | 0.1864 | 459,721 | 123,315 | |

[a] Irradiations were conducted in the Argonne National Laboratory CP-5 reactor at a neutron flux of $\sim 10^{13}$. Exposures were of twenty-four to forty-eight hours' duration.

[b] The calculated activity was based upon a 50-mg. sample for comparison purposes.

[c] The per cent error was calculated from the following formulas:

$$R.\,D. \times 100 = \text{Per cent error}$$

$$R.\,D. = \frac{Md}{N}$$

$$Md = \sqrt{M_1^2 + M_2^2}$$

$$M = \sqrt{\frac{\Sigma d^2}{n(n-1)}}$$

where: $R.\,D.$ = relative deviation, $Md$ = mean error of difference, $N$ = number of counts per unit time, $M_1$ = standard deviation of mean for sample, $M_2$ = standard deviation of mean for background, $M$ = standard deviation of mean, $\Sigma d^2$ = sum of the squares of the deviations from the arithmetic mean, and $n$ = number of observations.

[d] The values reported are the first set obtained. Sixteen determinations were performed to establish reproducibility and yielded an average error of 5.7%.

[e] These samples received an exposure of one hour in a neutron flux of $\sim 10^{13}$, in addition to forty-seven hours at $\sim 10^{6}$.

hours and conducted by counting all of the samples at the same energy level. The channel width was increased to "5" and the channel level set to the peak maximum of the gamma radiation (0.558 Mev. for arsenic-76 and 0.566 Mev. for antimony-122). Each sample was subjected to 10 five-minute counting sessions, background subtracted, and the count corrected for decay. In this manner, qualitative and quantitative studies were made on arsenic trioxide, carbarsone, oxophenarsine hydrochloride, antimony potassium tartrate, and stibophen. The results are compiled in Table I.

**Investigation of the Effect of Beta-Emitting Isotopes on the Gamma-Ray Scintillation Spectrometer.**—Preliminary investigations showed that beta particles, produced during irradiation of chemical samples, caused an increase in detectable activity. This activity interfered with gamma-ray counting at the 0.558 Mev. arsenic-76 radiation peak, when submitted to a scintillation spectrometer examination. The energy distribution of phosphorus-32,[11] a pure beta emitter, is presented in Fig. 1. In the 0.500 to 0.600 Mev. region, the activity ranged from 3,600 to 2,800 counts per minute. In the lower energy regions, the activity increased to 35,000 counts per minute. A shielding experiment was designed to determine the possibility of eliminating this interfering radiation. The investigation was performed beneath the cesium-137 radiation peak, utilizing a channel width of "1" and a gain of 1/4. The results are presented in Table II.

[11] Henceforth to be designated as P-32.

TABLE II.—RESULTS OF β-SHIELDING STUDY CONDUCTED WITH THE GAMMA-RAY SCINTILLATION SPECTROMETER

| | Activity, counts/min. | | | |
|---|---|---|---|---|
| Type of Shield[a] | Background[b] | Cesium-137[c] | Phosphorus-32[d] 200 μc.[e] | Cesium-137/Phosphorus-32 Activity Ratio[f] |
| None | 3 | 27,500 | 13,624 | 2.0 |
| Lucite[g] | 1 | 25,508 | 1,120 | 22.7 |
| Aluminum | 3 | 23,237 | 1,067 | 21.8 |
| Steel | 1 | 19,971 | 933 | 21.4 |
| Lead | 1 | 15,157 | 711 | 21.3 |

[a] All shields were constructed according to the same specifications (3/8 inch internal diameter × 1/8 inch external diameter) and had a minimum thickness of 3 mm.

[b] Background was determined with a 2-inch test tube containing 1.5 ml. of distilled water.

[c] This sample served as a calibration standard for the gamma-ray scintillation spectrometer. It emits two beta particles of 0.51 and 1.2 Mev. energies. The 2.6 minute half-life barium-137, a radioactive daughter, emits a 0.661 Mev. γ ray.

[d] Phosphorus-32 is a pure beta emitter of 1.71 Mev. energy.

[e] μc.—microcurie.

[f] This value was obtained by dividing the activity of cesium-137 by the activity of phosphorus-32 as determined under the conditions of this experiment.

[g] Lucite—E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.

Comparing the results of the lead shielding with those without shielding, a reduction in the cesium-137 radiation by one-third is noted. The P-32 activity was reduced by a factor greater than 10 when the lead shielding was applied. Background deter-

TABLE III.—THE DETECTION OF PHOSPHORUS-32 BETA PARTICLES AND BREMSSTRAHLUNG BY THE GAMMA-RAY SCINTILLATION SPECTROMETER

| | Phosphorus-32 Activity, μc.—Activity Detected, Counts/min.[a] | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 300 | 100 | 50 | 33.3 | 16.6 | 11.1 | 5.5 | 3.7 | 1.8 | 1.2 | 0.6 | 0.4 | 0.2 |
| Unshielded[b] | 22,394 | 7,998 | 3,627 | 3,968 | 1,131 | 877 | 481 | 262 | 121 | 114 | 51 | 24 | 15 |
| Shielded[c] | 2,215 | 451 | 222 | 144 | 56 | 37 | 22 | 12 | 6 | 4 | 4 | 3 | 2 |

Channel width 1; gain 1/4; 0.558 Mev. arsenic-76 radiation peak.

[a] Uncorrected for background. Each value is the average of 10 ten-minute counting sessions.
[b] Contained in two-inch test tubes.
[c] Contained in two-inch test tubes shielded by 3 mm. of lead.
Average background without shield, 5 counts per minute (average of 12 one-hour counting sessions).
Average background with shield, 2 counts per minute (average of 12 one-hour counting sessions).

minations were essentially the same with all of the shields.

The P-32 activity, observed in conjunction with the various shields, was attributed to Bremsstrahlung, since calculations indicate that 785 mg./cm.$^2$ of aluminum absorber will stop the 1.71 Mev. beta radiation. Three millimeters of lead corresponds to 3,860 mg./cm.$^2$.

Further work was conducted with the automatic recording attachment to study the effect of Bremsstrahlung resulting from P-32 activity. Quantitative results (Table III) were determined by counting under the 0.558 Mev. arsenic-76 radiation peak. Activity resulting from P-32 was reduced to background at the ~0.2 to ~0.4 μc. level, using the lead shield. At the ~0.2 μc. level, without the lead shield, the P-32 activity was three times greater than the average background activity. These experiments show the level of detection of P-32 Bremsstrahlung by the gamma-ray scintillation spectrometer and prove the fact that the presence of beta particles at microcurie levels will interfere with quantitative gamma counting.

## SUMMARY AND CONCLUSIONS

1. This investigation was conducted to show the applicability of slow neutron activation to the analysis of pharmaceuticals. Irradiations were performed at a neutron flux of ~$10^6$, in the case of pure compounds, to emphasize the possibilities of laboratory sources for routine analyses.

2. The gamma-ray scintillation spectrometer, utilized in this investigation, was found to be sensitive to microcurie amounts of beta-emitting isotopes. Three millimeters Lucite, aluminum, steel, and lead shields were constructed to reduce the beta interference, but they were ineffective in completely controlling the Bremsstrahlung.

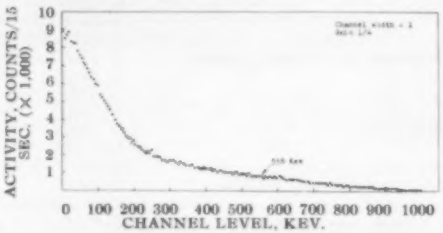


Fig. 1.—Phosphorus-32 (50 μc.) energy distribution.

3. The method developed was applied to the analysis of pure samples of arsenic trioxide, carbarsone, oxophenarsine, antimony potassium tartrate, and stibophen. Within the milligram level, the average error was between 4 and 5 per cent. A higher neutron flux and/or a longer irradiation time would result in sensitivities at the microgram and submicrogram levels. The primary advantage of the procedure is the minimum amount of chemical manipulation required..

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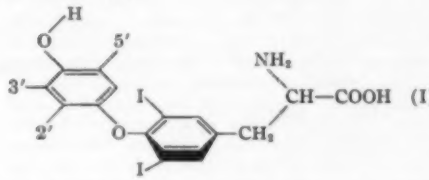
# Thyroxine Analogs II*

## Synthesis of 3,5-Diiodo-4-(2′,3′-dimethylphenoxy)-DL-phenylalanine and Its 4′-Hydroxy Congener

By EUGENE C. JORGENSEN and PUSHKAR N. KAUL

**The synthesis of thyroxine analogs, 3,5-diiodo - 4 - (2′,3′ - dimethylphenoxy) - DL-phenylalanine and 3,5-diiodo-2′,3′-dimethyl-DL-thyronine is described. These compounds were prepared in order to study the effect on biological response of alkyl substituents in sterically oriented analogs of thyroxine. Both compounds displayed thyromimetic activity.**

SINCE THE ORIGINAL SYNTHESIS of thyroxine by Harington and Barger (1), many analogs have been prepared in attempts to establish structural requirements for thyroxine-like activity. Recently (2), attention has been directed to a potential steric factor whereby the 3′ and 5′-positions of 3,5-diiodothyronine and related diphenyl ethers become nonequivalent in their spatial orientation following introduction of a 2′-alkyl substituent (I).




(I)

The 2′-substituent assumes a preferred orientation distal to the alanine-bearing ring, thus providing a minimal interaction between the 2′-substituent and the bulky 3,5-iodines and the ring bearing them. Substituent groups on the prime ring may be located spatially by reference to their positions relative to the orienting 2′-alkyl group, and thus to the diiodophenylalanine ring.

Zenker and Jorgensen (2) have reported the synthesis of a series of 3,5-diiodo-4-(2′-alkylphenoxy)-DL-phenylalanines bearing additional 4′-hydroxyl and 5′-methyl substituents. These compounds reflect the orientation of a 5′-alkyl group in a position proximal to the diiodophenylalanine ring. Analogous compounds carrying 4′-hydroxyl and 3′,5′-dimethyl substituents have been shown to possess thyroxine-like activity (3, 4). For this reason, it was desirable to extend the series to provide a 3′-methyl group, oriented distal to the diiodophenylalanine ring as is the case for 3,5-diiodo-2′,3′-dimethyl thyronine (VI). Since the 5′-position was available for substitution, it was considered desirable to prepare the triiodo analog of this series, 3,5,5′-triiodo-2′,3′-dimethyl-DL-thyronine (VII). Analogs lacking the 4′-hydroxyl group have been reported (5) to act as thyroxine antagonists. The related compound, 3,5-diiodo-4-(2′,3′-dimethylphenoxy)-DL-phenylalanine (V), was therefore prepared to provide a distally oriented 3′-methyl group in this series of potential antagonists.

The synthetic route used was that developed by Hems and co-workers (6). 3,5-Dinitro-4-(*p*-toluenesulfonyloxy) - DL - phenylalanine - N - acetyl ethyl ester (II) was allowed to condense in the presence of pyridine with 2,3-dimethylphenol, yielding 3,5-dinitro-4-(2′,3′-dimethylphenoxy)-DL-phenylalanine-N-acetyl ethyl ester (IIIa) or with 2,3-dimethyl-4-methoxyphenol yielding 3,5-dinitro - 4 - (2′,3′ - dimethyl - 4′ - methoxyphenoxy)-DL-phenylalanine-N-acetyl ethyl ester (IIIb).

These dinitrodiphenol esters were hydrogenated to the diamines with Pd-on-charcoal catalyst which, without isolation, were tetrazotized and converted to the corresponding 3,5-diiodo analogs. Hydrolysis with hydrochloric and acetic acids converted 3,5-diiodo-4-(2′,3′-dimethylphenoxy)-DL-phenylalanine-N-acetyl ethyl ester (IVa) to 3,5-diiodo-4-(2′,3′-dimethylphenoxy)-DL-phenylalanine (V). To effect cleavage of the methoxyl group in 3,5-diiodo-4-(2′,3′-dimethyl-4′ - methoxyphenoxy) - DL - phenylalanine - N - acetyl ethyl easter (IVb), the compound was treated with hydriodic acid in acetic acid, yielding the desired 3,5-diiodo-2′,3′-dimethyl thyronine (VI). Attempts to form 3,5,5′-triiodo-2′,3′-dimethyl-DL-thyronine (VII) by the iodination of VI with iodine and potassium iodide in aqueous ethylamine were unsuccessful.

### BIOLOGICAL RESULTS

3,5 - Diiodo -2′,3′ - dimethyl - DL - thyronine (VI) demonstrated thyroxine-like activity in the rat

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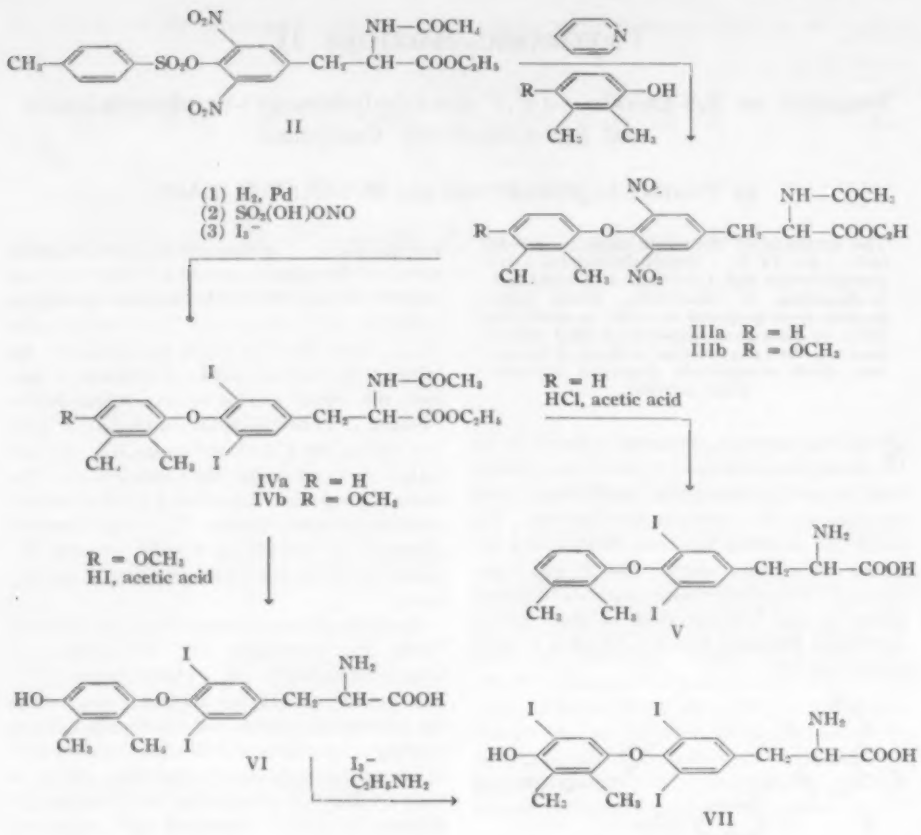


at 3 mg./Kg./day for ten days in the antigoiter assay of Cortell (7) and at a single dose of 20 mg./Kg. in the oxygen consumption assay of Holtkamp and Heming (8). 3,5-Diiodo-4-(2′,3′-dimethylphenoxy) -DL-phenylalanine (V) was active at 7 mg./Kg./day for ten days in the antigoiter assay, but inactive in the test of oxygen consumption at a single dose of 20 mg./Kg. This represents the first report of a thyromimetic thyroxine analog unsubstituted in the 4′-position. A detailed discussion of these results relating stereochemical orientation of substituents to biological response will be submitted elsewhere.

## EXPERIMENTAL[1]

**3,5 - Dinitro - 4 - (*p* - toluenesulfonyloxy) - DL-phenylalanine-N-acetyl Ethyl Ester (II).**—Crystallized from aqueous acetone, m. p. 160–162°, prepared in 75% yield from 3,5-dinitro-DL-tyrosine-N-acetyl ethyl ester[2] by the method of Barnes, *et al.* (5), who reported m. p. 157–158°.

**2,3-Dimethyl-1,4-hydroquinone.**—Prepared from 2,3-dimethylphenol[3] by the method of Smith and Tess (9) in 36% yield. Crystallized from benzene and sublimed at 130° (17 mm.), m. p. 219–220° (decompn.). Lit. (9), 223–224° (decompn.).

*Anal.*—Calcd. for $C_8H_{10}O_2$ (138.2): C, 69.55; H, 7.30. Found: C, 69.63; H, 7.16.

**2,3-Dimethyl-4-methoxyphenol.**—To a well-stirred solution of 2,3-dimethyl-1,4-hydroquinone (62.3 Gm., 0.45 mole) in sodium hydroxide (44.8 Gm., 1.12 moles) and water (350 ml.) at 10°, dimethylsulfate (68 Gm., 0.54 mole) was added in small portions over a period of two hours, and stirring continued an additional hour at 10° (10). The mixture was extracted with five 100-ml. portions of ether (Extract I), the aqueous solution was acidified with 6 *N* hydrochloric acid and extracted with four 100-ml. portions of ether (Extract II). Following removal of ether, Extract I yielded 34.0 Gm. of 2,3-dimethyl-1,4-dimethoxybenzene, m. p. 78–79°. Lit. (11), 78°. The residue following ether evaporation of Extract II was extracted repeatedly with boiling ligroin (30–60°). The combined ligroin extracts were evaporated and the residue crystallized from water, yielding 12.0 Gm. (18%) of 2,3-dimethyl-4-methoxyphenol, m. p. 95–96°. Lit. (9), 95–97°.

**3,5 - Dinitro - 4 - (2′,3′ - dimethylphenoxy) - DL-phenylalanine-N-acetyl Ethyl Ester (IIIa).**—3,5-Dinitro - 4 - (*p*-toluenesulfonyloxy) - DL - phenylala-

[1] Melting points obtained on Fisher-Johns melting point apparatus and are uncorrected. Microanalysis by the Microanalytical Laboratory, Department of Chemistry, University of California.
[2] Prepared by Mr. Robert Day.
[3] Aldrich Chemical Co., Milwaukee, Wis.

nine-N-acetyl ethyl ester (16.5 Gm., 0.033 mole) was dissolved in dry chloroform (100 ml.) and heated under reflux with dry pyridine (20 ml., 0.25 mole) for thirty minutes. 2,3-Dimethylphenol[4] (8.1 Gm., 0.066 mole) was added and the mixture heated at reflux temperature for an additional three hours. After cooling, the chloroform solution was washed successively with 2 *N* hydrochloric acid, water, 2 *N* sodium hydroxide, and water, dried over calcium chloride, filtered, and the chloroform removed under reduced pressure. The residue was crystallized from ethanol, yielding 3.6 Gm. (25%), m. p. 134–135°.

*Anal.*—Calcd. for $C_{21}H_{22}N_2O_8$ (445.4): C, 56.62; H, 5.21. Found: C, 56.44; H, 5.28.

**3,5 - Diiodo - 4 - (2′,3′ - dimethylphenoxy)-DL-phenylalanine-N-acetyl Ethyl Ester (IVa).**—3,5-Dinitro - 4 - (2′,3′ - dimethylphenoxy) - DL - phenylalanine-N-acetyl ethyl ester (4.45 Gm., 0.01 mole) dissolved in glacial acetic acid (200 ml.) was shaken for twenty minutes in the presence of Pd-on-charcoal (10%, 2.5 Gm.) and hydrogen (initial pressure 38 p. s. i.). Hydrogen uptake stopped at 96% of theoretical. Concentrated sulfuric acid (15 ml.) was added, the catalyst removed by filtration through Celite and concentrated sulfuric acid (75 ml.) added with cooling to the filtrate. The resulting solution was added over two hours to a stirred mixture of nitrosylsulfuric acid [sodium nitrite (2.1 Gm., 0.03 mole) added in small portions to sulfuric acid (200 ml.) at 50°, then diluted at 0° with glacial acetic acid (200 ml.)] at −5°. The tetrazonium mixture was stirred an additional one and one-half hours at 0°, then added to a well-stirred mixture at 25° of iodine (23 Gm., 0.09 mole) and sodium iodide (23 Gm., 0.14 mole) diluted with water (1,000 ml.) and underlayed with chloroform (500 ml.). After two hours, the chloroform phase was separated and the aqueous layer extracted with chloroform. The combined chloroform extracts were washed with 0.5 *N* sodium metabisulfite and water, dried over calcium chloride, and the chloroform removed under reduced pressure. The residue was dissolved in dry chloroform (20 ml.), passed through an alumina column (acid washed, 13 × 2.0 cm.), the eluate evaporated, and the residue crystallized from alcohol. Yield 3.5 Gm. (58%), m. p. 130–131°.

*Anal.*—Calcd. for $C_{21}H_{22}I_2NO_4$ (607.3): C, 41.53; H, 3.82. Found: C, 41.85; H, 3.88.

**3,5 - Diiodo - 4 - (2′,3′ - dimethylphenoxy)-DL-phenylalanine (V).**—3,5-Diiodo-4-(2′,3′-dimethylphenoxy)-DL-phenylalanine-N-acetyl ethyl ester (3.0 Gm., 0.005 mole) was heated under reflux for one hour with glacial acetic acid (15 ml.) and concentrated hydrochloric acid (15 ml.). The cooled mixture was diluted with water to 100 ml., 6 *N* sodium hydroxide (30 ml.) was added, then adjusted carefully to pH 5.0 with 1 *N* sodium hydroxide. The flocculent precipitate was collected by centrifugation, washed thoroughly with water, and dried *in vacuo*. Crystallization from aqueous acetic acid yielded 2.4 Gm. (90%) of V, m. p. 197–199° (decompn.).

*Anal.*—Calcd. for $C_{17}H_{17}I_2NO_5$ (537.3): C, 38.01; H, 3.19; I, 47.25. Found: C, 37.88; H, 3.55; I, 47.02.

**3,5 - Dinitro - 4 - (2′,3′ - dimethyl - 4′ - methoxyphenoxy) - DL - phenylalanine - N - acetyl Ethyl Ester (IIIb).**—3,5-Dinitro-4-(*p*-toluenesulfonyloxy)-DL-phenylalanine-N-acetyl ethyl ester (52.0 Gm., 0.104 mole) was dissolved in dry chloroform (100 ml.) and heated under reflux with dry pyridine (16 ml., 0.2 mole) for forty-five minutes. 2,3-Dimethyl-4-methoxyphenol (8.0 Gm., 0.052 mole) was added and the mixture heated at reflux temperature for an additional three hours. After cooling, the chloroform solution was washed successively with 2 *N* hydrochloric acid, water, 2 *N* sodium hydroxide, and water, dried over calcium chloride, and the chloroform removed under reduced pressure. The residue was crystallized from ethanol, yielding 4.0 Gm. (50%), m. p. 142–143°.

*Anal.*—Calcd. for $C_{22}H_{25}N_3O_4$ (475.4): C, 55.57; H, 5.30. Found: C, 55.84; H, 5.16.

**3,5 - Diiodo - 4 - (2′,3′ - dimethyl - 4′ - methoxyphenoxy) - DL - phenylalanine - N - acetyl Ethyl Ester (IVb).**—3,5 - Dinitro - 4 - (2′,3′ - dimethyl - 4′-methoxyphenoxy)-DL-phenylalanine-N-acetyl ethyl ester (5.0 Gm., 0.011 mole) was converted to the diiodo derivative by a procedure identical with that used in the synthesis of 3,5-diiodo-4-(2′,3′-dimethylphenoxy) - DL - phenylalanine - N - acetyl ethyl ester (IVa). Crystallization from absolute alcohol–acetone (4:1) yielded 3.5 Gm. (52%), m. p. 196–198°.

*Anal.*—Calcd. for $C_{22}H_{23}I_2NO_5$ (637.3): C, 41.46; H, 3.95. Found: C, 41.64; H, 4.02.

**3,5 - Diiodo - 2′,3′ - dimethyl - DL - thyronine (VI).**—3,5 - Diiodo - 4 - (2′,3′ - dimethyl - 4′ - methoxyphenoxy)-DL-phenylalanine-N-acetyl ethyl ester (1.5 Gm., 0.0025 mole) was heated under reflux for four hours with glacial acetic acid (15 ml.) and hydriodic acid (58%, 30 ml.). The reaction mixture was distilled to near dryness from a water bath at 50° (5 mm.). The residue was dissolved in a suspension of sodium metabisulfite in hot ethanol, and the solution adjusted to pH 5.0 with 2 *N* sodium acetate. The precipitate was collected by centrifugation, dissolved, and precipitated three times from 1 *N* sodium hydroxide by adjustment to pH 5.0 with 2 *N* hydrochloric acid and a few drops of aqueous sodium acetate, washed by suspension in water, and collected by centrifugation. Drying at 100° (2 mm.) over phosphorus pentoxide yielded 0.85 Gm. (65%) of a colorless hygroscopic powder, m. p. 223–225° (decompn.).

*Anal.*—Calcd. for $C_{17}H_{17}I_2NO_4$ (553.2): C, 36.91; H, 3.10; I, 45.89. Found: C, 37.27; H, 3.09; I, 46.12.

[4] Aldrich Chemical Co., Milwaukee, Wis.

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# The Pharmacy of Chlorothiazide (6-Chloro-7-sulfamyl-1,2,4-benzothiadiazine-1,1-dioxide): A New Orally Effective Diuretic Agent*

By WALTER F. CHARNICKI†, FREDERICK A. BACHER, STEWART A. FREEMAN, and DONALD H. DeCESARE

**Chlorothiazide occurs as a white, crystalline powder which can be formulated in tablets or suspension for use as an oral diuretic. It is very slightly soluble in water and is soluble but unstable in alkaline solutions. The solid sodium salt is stable and can be dissolved in water for injection to give a suitable intravenous dosage form. Chlorothiazide's absorption maximum at 292 mμ in 0.1 *N* sodium hydroxide can be used for analysis of all dosage forms.**

A RECENT STUDY of aromatic sulfonamides has led to the synthesis of benzothiadiazine derivatives (1). Pharmacological studies have revealed that this series of compounds possesses diuretic activity of rather unusual quality. In animals they not only promote the excretion of sodium but also of chloride ions (2, 3, 4). The compound selected for clinical trial from this series was 6-chloro-7-sulfamyl-1,2,4-benzothiadiazine-1,1-dioxide, known generically as chlorothiazide (Diuril). This derivative belongs to a class of heterocyclic compounds of which the parent compound is 1,2,4-benzothiadiazine.

Clinical studies in man have substantiated the earlier pharmacological findings and confirmed the unusual ability of chlorothiazide to promote the excretion of sodium and chloride, and to a smaller extent, potassium chloride. This suggested that it be designated a saluretic agent (5, 6, 7). More recently it has been observed that chlorothiazide produces significant reduction of blood pressure in hypertensive but not normotensive patients. Furthermore, it potentiates the antihypertensive action of drugs such as reserpine, veratrum alkaloids, hydralazine, and ganglionic blocking agents, and also enhances the hypotensive effects of surgical sympathectomy (8–11).

The mode of action of chlorothiazide whereby it enhances the excretion of sodium and chloride has not been fully defined. *In vitro* studies have shown the compound to have carbonic anhydrase inhibitory action which seems to be relatively specific for the renal tubular mechanism.

Chlorothiazide has a high therapeutic index and a low inherent toxicity. In human bioassays, as judged by enhancement of sodium excretion, chlorothiazide in a dose of 0.25 Gm. orally has an effect comparable to that of the classic carbonic anhydrase inhibitors. An oral dose of 0.5 to 1.0 Gm. of chlorothiazide is comparable to the oral administration of mercurial diuretics, and an oral dose of 1.0 to 2.0 Gm. to parenteral mercurials. Chlorothiazide thus has wide application.

The onset of action is within two hours following the oral administration of chlorothiazide with maximal effect at about four hours. The major saluretic effect lasts approximately six to twelve hours.

It is of interest to note that following intravenous administration of chlorothiazide the onset of action is within fifteen minutes, the maximal effect being obtained within thirty minutes. The duration of effect is relatively short; the major response lasts about two hours. The total effect upon electrolyte and water excretion is thus brief and quantitatively less than that of a comparable oral dose.

The usual adult dosage range is one or two 0.5-Gm. tablets of chlorothiazide once or twice a day. When the total daily dose is 1.0 Gm. or more it is usually administered in two divided doses at an interval of six to twelve hours.

The clinical reports received to date attesting to the outstanding value of chlorothiazide as a safe and effective agent in the treatment of edematous states as well as in the treatment of hypertensive vascular diseases have prompted us to describe the physical properties and pharmaceutical dosage forms of this new diuretic agent.

## PHYSICAL PROPERTIES

Chlorothiazide is a white crystalline compound melting about 355° with decomposition. Its empirical formula is $C_7H_6ClN_3O_4S_2$ and the molecular weight is 295.7. It may be represented structurally as follows:

* Received June 5, 1959, from the Merck Sharp & Dohme Research Laboratories, Division of Merck & Co., Inc., West Point, Pa.

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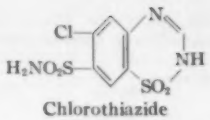


Chlorothiazide

Chlorothiazide is very slightly soluble in water and glycerin. It is practically insoluble in ether, chloroform, and benzene. One gram dissolves in about 300 ml. of acetone, 500 ml. of 95% ethanol, 350 ml. of methanol, 150 ml. of pyridine, or 9 ml. of dimethylformamide. The solubility of chlorothiazide in water or dilute acids is approximately 0.2 mg. per ml. At pH 7 the solubility is approximately 0.7 mg. per ml. and at pH 8.5 it is 16 mg. per ml. More than 1 Gm. will dissolve in 10 ml. of 1 *N* sodium hydroxide.

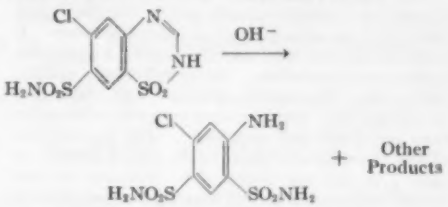
Chlorothiazide is a weak dibasic acid with $pK_1 = 6.7$ and $pK_2 = 9.5$. In acid solution it shows an ultraviolet absorption maximum at 278 mμ, with $a$ (1%, 1 cm.) approximately 420. In 0.1 *N* sodium hydroxide the absorption maximum occurs at 292 mμ, with $a$ (1%, 1 cm.) approximately 430.

The heterocyclic ring of chlorothiazide is slowly hydrolyzed in alkaline solutions yielding a diazotizable amine. At room temperatures, solutions in 0.1 *N* sodium hydroxide hydrolyze at the rate of approximately 1% per hour while at 100° the initial rate is about 2% per minute. While the stability of chlorothiazide in neutral or acidic solutions is substantially greater, the low solubility of the drug at lower pH's precludes the attainment of physiologically effective concentrations.

## TYPICAL FORMULATIONS

**Compressed Tablets.**—Stability studies have indicated that compressed tablets of chlorothiazide are satisfactory when manufactured by either the wet granulating or by "slugging" techniques. Tablets containing 250 mg. and 500 mg. of chlorothiazide and corn starch, and granulated with starch paste have been made with a hardness of 7 to 8 Kg. and a disintegration time of five minutes at 37°. Tablets containing 250 mg. of chlorothiazide and additives such as polyethylene glycol 4000 and methylcellulose to increase hardness have been made by slugging. These usually have a hardness of 4 to 5 Kg. and a disintegration time of five minutes at 37°. Hardness was determined with a Monsanto hardness tester and disintegration was taken with a Stoll-Gershberg type of apparatus.

**Injection.**—Chlorothiazide is uniformly and well absorbed when given orally. However, when oral administration is not possible or desirable, chlorothiazide may be administered intravenously. The dosage form of chlorothiazide for intravenous use requires the chlorothiazide to be either in solution or in a form completely and quickly soluble. Chlorothiazide is somewhat soluble in polyethylene glycol 300 and polyethylene glycol 400. It is readily soluble in dilute sodium hydroxide solutions. The latter solutions, unfortunately, decompose upon standing at room temperature or elevated temperatures with hydrolysis of the heterocyclic ring. The reaction may be summarized as follows:



Because of this instability sterile freeze-dried solids were investigated for parenteral use in clinical studies. Aqueous solutions of the monosodium salt of chlorothiazide freeze-dry to yield hard shrunken plugs with a poor appearance and which dissolve very slowly upon adding water. This difficulty was overcome by the addition of suitable excipients for lyophilization, particularly dry sugar alcohols. Freeze-dried solids containing 0.5 Gm. of chlorothiazide as the sodium salt can be restored with 10 ml. or more of water for injection to give a clear solution having a pH of 9.5. When a vial containing 0.5 Gm. of chlorothiazide (as the sodium salt) is restored with 20 ml. of water, the resulting solution is practically isotonic and has a freezing point of −0.505°.

Vials of freeze-dried chlorothiazide may also be restored with normal saline and 5% dextrose.

TABLE I.—COMPATIBILITY OF CHLOROTHIAZIDE WITH HYPOTENSIVE AGENTS

| Drug[a] | pH | Amount Added,[b] ml. | pH of Mixture | Appearance[c] Initially | Appearance[c] After 24 Hr. |
|---|---|---|---|---|---|
| Protoveratrines A and B | 5.9 | 1.2 | 9.1 | S | S |
| Pentolinium tartrate | 5.3 | 1.0 | 9.1 | S | S |
| Hydralazine hydrochloride | 3.7 | 2.0 | 9.0 | H | P |
| Cryptenamine acetate | 4.7 | 1.0 | 9.2 | S | S |
| Alkavervir | 3.2 | 1.0 | 9.0 | S | S |
| Reserpine | 3.0 | 1.0 | 9.0 | H | P |
| Mecamylamine hydrochloride | 7.5 | 1.0 | 9.2 | S | S |
| Cryptenamine acetate plus hydralazine hydrochloride | 4.7<br>3.7 | 1.0<br>2.0 | 9.0 | H | P |
| Reserpine plus hydralazine hydrochloride | 3.0<br>3.7 | 1.0<br>2.0 | 8.8 | H | P |
| Reserpine plus mecamylamine HCl | 3.3<br>7.5 | 0.5<br>1.0 | 9.3 | H | P |

[a] Hypotensive agent in commercially available solution.
[b] The indicated volume of solution was added to 125 ml. of 0.4% chlorothiazide solution in 5% dextrose. The pH of this solution was 9.15.
[c] S—Solution clear with no discoloration or precipitate. H—Solution became cloudy and developed color. P—Precipitate developed.

These diluents do not alter the pH and therefore result in clear solutions. On the other hand, diluents which lower the pH of the restored solution to pH 8.8 or less tend to yield cloudy solutions due to precipitation of chlorothiazide free acid.

A 10% solution of chlorothiazide free acid may be prepared in a 75% aqueous solution of polyethylene glycol 300 or 400. Although this solution is effective as a diuretic when administered intravenously to the dog, clinical studies were not conducted.

**Compatibility of Sodium Chlorothiazide Solutions With Hypotensive Drugs.**—The potentiating action of chlorothiazide upon such hypotensive drugs as reserpine, veratrum alkaloids, hydralazine, and ganglionic blocking agents led us to study the compatibility of chlorothiazide sodium solution with some of these agents. The results of this study are shown in Table I.

**Oral Fluid Preparation.**—Chlorothiazide free acid is only very slightly soluble and is therefore quite stable in aqueous suspension at pH 6.5 or lower. It is also compatible with such materials as tragacanth, carboxymethylcellulose, methylcellulose, gelatin, and acacia. Suspensions prepared with tragacanth in a syrup base were found to be quite satisfactory both physically and chemically. The particle size of the chlorothiazide used in the suspensions averaged 5 μ and the preparations resuspended very readily with no evidence of caking even after storage at higher temperatures. They were readily flavored to mask the slight bitterness of the chlorothiazide by the addition of citrus flavors.

**Stability.**—The stability of chlorothiazide in the formulations described was evaluated over prolonged intervals at widely varying temperatures. The excellent stability of chlorothiazide in these products has been summarized in Tables II, III, and IV.

## ANALYTICAL PROCEDURES

Chlorothiazide can be assayed by ultraviolet absorption at 292 mμ after diluting the sample with 0.1 *N* sodium hydroxide, filtering if necessary, and then diluting to approximately 1 mg. per 100 ml. for measurement. If the measurement is completed within one hour, alkaline decomposition is insignificant. Many neutral or basic interferences can be removed by washing an alkaline solution with chloroform before final dilution. Acidic or phenolic substances can usually be removed by washing an intermediate acidic dilution (less than 30 mg./100 ml.) with chloroform before final dilution with 0.1 *N* sodium hydroxide.

While the major hydrolysis product of chlorothiazide (see reaction for decomposition) is not removed by the above treatments, the absorption of this decomposition product at 292 mμ is only 25% that of chlorothiazide. For stability studies in which apparent decomposition (as determined by ultraviolet absorption) is less than 10%, no significant error is introduced if the absorption due to the amine is ignored. The hydrolysis product itself can be determined by diazotization and coupling by a modified Bratton-Marshall method (12).

**Compressed Tablets.**—Weigh accurately powdered tablets equivalent to 250 mg. of chlorothiazide into a 250-ml. volumetric flask. Add about 200 ml. 0.1 *N* sodium hydroxide solution and shake for fifteen minutes. Dilute to volume with sodium hydroxide solution, mix thoroughly, and filter about 50 ml. through a No. 2 Whatman paper discarding the first 25 ml. of filtrate. Dilute 1 to 100 ml. with 0.1 *N* sodium hydroxide and determine absorbance at 292 mμ. Calculate using $a$ (1%, 1 cm.) = 430.

**Freeze-Dried Sodium Salt.**—Dissolve the contents of one vial (approx. 500 mg.), transfer quantitatively to a 250-ml. volumetric flask with 0.1 *N* sodium hydroxide, and dilute to volume. Dilute this further with 0.1 *N* sodium hydroxide; 25 ml. to 500 ml. and 10 ml. of the latter solution to 100 ml. Determine absorbance at 292 mμ and calculate using $a$ (1%, 1 cm.) = 430.

**Oral Suspension.**—Dilute 5 ml. of suspension to 250 ml. with 0.1 *N* sodium hydroxide (solution A). Dilute 10 ml. of solution A to 100 ml. with 0.1 *N*

TABLE II.—STABILITY OF CHLOROTHIAZIDE IN TABLET FORMULATIONS

| Formulation | Storage Temp., °C. | Storage Period | Assay, % Label Claim |
|---|---|---|---|
| C.T. 500 mg.[a] | | Initial | 101 |
| | 80 | 5 days | 99 |
| | 70 | 10 days | 101 |
| | 60 | 20 days | 99 |
| | 37 | 2 mo. | 101 |
| | | 4 mo. | 98 |
| | | 6 mo. | 98 |
| | | 13 mo. | 98 |
| | RT | 4 mo. | 99 |
| | | 6 mo. | 99 |
| | | 13 mo. | 101 |
| C.T. 250 mg.[b] | | Initial | 99 |
| | 50 | 1 mo. | 94 |
| | 37 | 7½ mo. | 99 |
| | RT | 4½ mo. | 99 |
| | | 7½ mo. | 100 |

[a] Prepared by wet granulation.
[b] Prepared by slugging.

TABLE III.—STABILITY OF CHLOROTHIAZIDE IN FREEZE-DRIED PREPARATION

| Storage Temp., °C. | Storage Period | Assay, % Label Claim |
|---|---|---|
| | Initial | 98 |
| 60 | 2 wks. | 94 |
| 50 | 2 wks. | 98 |
| | 5 mo. | 95 |
| | 14 mo. | 99 |
| 37 | 1 mo. | 96 |
| | 5 mo. | 98 |
| | 14 mo. | 99 |
| RT | 2 mo. | 101 |
| | 5 mo. | 100 |
| | 14 mo. | 100 |

TABLE IV.—STABILITY OF LYOPHILIZED CHLOROTHIAZIDE 500 MG. AFTER RESTORATION

| Diluent | Volume, ml. | Assay, % Initial Concentration — Time After Restoration, Hr. — 0 | 3 | 22 | 48 | 72 |
|---|---|---|---|---|---|---|
| Water for injection | 10 | 100 | 99.5 | 98 | 97.5 | 98 |
| Dextrose 5% | 10 | 100 | 99.5 | 98 | 97 | 96 |
| Dextrose 5% | 500 | 100 | 99.5 | 98 | 94 | 94 |

hydrochloric acid (solution B). Wash about 50 ml. of solution B with 2 × 25 ml. chloroform. Dilute 10 ml. of washed solution B to 100 ml. with 0.1 *N* sodium hydroxide. Read absorbance at 292 mμ and calculate using *a* (1%, 1 cm.)=430.

## SUMMARY

1. Methods were developed for the preparation of compressed tablets, suspension, and freeze-dried solid of chlorothiazide.

2. Analytical procedures were developed for the assay of chlorothiazide in these dosage forms.

3. The chlorothiazide in these dosage forms has been found to be quite stable in both accelerated and long term stability studies.

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# Alkaloids of *Vinca rosea* Linn. (*Catharanthus roseus* G. Don.) V*

## Preparation and Characterization of Alkaloids

By GORDON H. SVOBODA, NORBERT NEUSS, and MARVIN GORMAN

**As part of continuing study of alkaloid-bearing members of the family *Apocynaceae*, we have undertaken a detailed phytochemical investigation of the pantropical plant *Vinca rosea* Linn. (1). Twelve crystalline compounds have been obtained and characterized. Details of the procedure leading to the preparation of pure substances are described. Two of these alkaloids, leurosine and vincaleukoblastine, possess activity against P-1534 leukemia in mice.**

THE APOCYNACEOUS plant *Vinca rosea* Linn. has enjoyed a wide reputation in folk medicine as an oral hypoglycemic agent[1] (2, 3). As part of our general program of investigation of plants in this family (4), a phytochemical study of this plant was undertaken. Various alkaloidal and nonalkaloidal fractions were tested for their ability to lower blood sugar. In our hands, however, these results were all negative (5).[2] During the testing, the observation was made that certain fractions prepared from the crude drug produced a delayed toxicity in experimental animals. Subsequently these extracts were submitted for testing against experimental leukemia (10). Limited prolongation of life was observed in DBA-2 mice against P-1534 leukemia with certain preparations of plant material.

This activity prompted a systematic fractionation of extracts in a search for the active principles free of extraneous, undesirable toxic substances. Initial testing soon indicated that the biologically active entities were confined to the complex alkaloidal portion of the drug.

In this communication a procedure is described for the preparation of leurosine and vincaleukoblastine,[3] two alkaloids possessing the anti-

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We wish to acknowledge and thank the following individuals for their aid in the course of this investigation: Botanical classification: Dr. Julian Steyermark, Research Associate, Missouri Botanical Gardens. Plant processing: Mr. A. J. Barnes, Jr. Laboratory assistance: Mrs. Nancy J. Cone, Miss Ruthanne Miller, Messrs. R. J. Armstrong, A. T. Oliver, and George Johnson. Physical data: Miss Ann Van Camp, Dr. H. E. Boaz, Mr. L. G. Howard, and associates. Microanalyses: Messrs. G. M. Maciak, W. L. Brown, H. L. Hunter, and R. Hughes. Biological evaluations: Drs. M. Root and I. S. Johnson and associates. Paper chromatography: Mr. H. Bird. Plant procurement: Ellis Meer Corp., New York, N. Y. Alkaloid samples: Dr. C. T. Beer, University of Western Ontario, London, Canada; authentic sample of vincaleukoblastine sulfate. Professor Janot and Dr. Jean Le Men, University of Paris, Faculty of Pharmacy, Paris, France; sample of vindolinine dihydrochloride.

Presented to the Scientific Section, A. PH. A., Cincinnati meeting, August 1959.

[1] In an earlier description of medicinal properties of this plant, Peckolt has mentioned that the infusion of the leaves of *Vinca rosea* Linn. is used in Brazil against hemorrhage, scurvy, as a mouthwash for toothache, and for healing and cleaning of chronic wounds (6). Most recently Chopra, *et al.*, have reported that the total alkaloids possess a limited antibiotic activity (against *V. cholera* and *M. pyogenes* var. *aureus*) as well as a significant and sustained hypotensive action (6a).

[2] A literature search revealed that earlier workers have investigated the properties of this plant on carbohydrate metabolism with essentially negative results (7, 8, 9).

[3] After the preparation of leurosine by one of us (G. H. S.), it was learned that Drs. Noble, Beer, and Cutts of the Department of Medical Research, University of Western Ontario, London, Canada, have also observed hematological responses in certain preparations of *Vinca rosea* Linn. leaves and subsequently have obtained an active alkaloid, vincaleukoblastine. Since that time, certain phases of the work on vincaleukoblastine have been carried out in cooperation with the Canadian group.

Fig. 1.—*Vinca rosea* Linn.

leukemic activity mentioned above. Pertinent data regarding other crystalline alkaloids (Tables I and II) obtained from this plant are also presented.

## BOTANICAL AND CHEMICAL CHARACTERISTICS

**Botanical Description of Vinca rosea Linn.**—*Vinca rosea* Linn. is an erect, everblooming pubescent herb or subshrub, one to two feet high (Fig. 1) (11). It is generally cosmopolitan in the tropics but is also cultivated widely in gardens throughout the world (12). Two color varieties (pink and white) are found in the natural state and an additional three (blush pink with red eye, crimson, and white with red eye) are listed as commercially available seed hybrids (13). Generally accepted synonyms for the genus *Vinca* are *Pervinca*, *Lochnera*, and *Catharanthus*.[4] The genus is placed in the group *Lochnerineae* of the tribe *Alstoniae*, of the subfamily *Plumeroideae*.[4]

[4] Pichon, an authority in the taxonomy of the *Apocynaceae* has shown, however, that the genera *Vinca* and *Lochnera* differ in thirty-four morphological characteristics and therefore should not be used as synonyms. He also found that the name *Lochnera Reichb.* was predated some seven months by the name *Catharanthus* G. Don. Therefore, the correct botanical name for this plant is *Catharanthus roseus* G. Don (14), but to avoid confusion we shall continue to use the more common *Vinca rosea* Linn. A complete botanical description and bibliography on the genus *Vinca* Linn. is given in the doctoral thesis of Marcelle Gabbai "Les Alcaloides des Parvenches *Vinca et Catharanthus (Apocynaceae)*," Université de Paris, Faculté de Pharmacie, Serial No. U-291, 1958, Paris, France. We thank Dr. Jean Le Men of this university for making the thesis available to us.

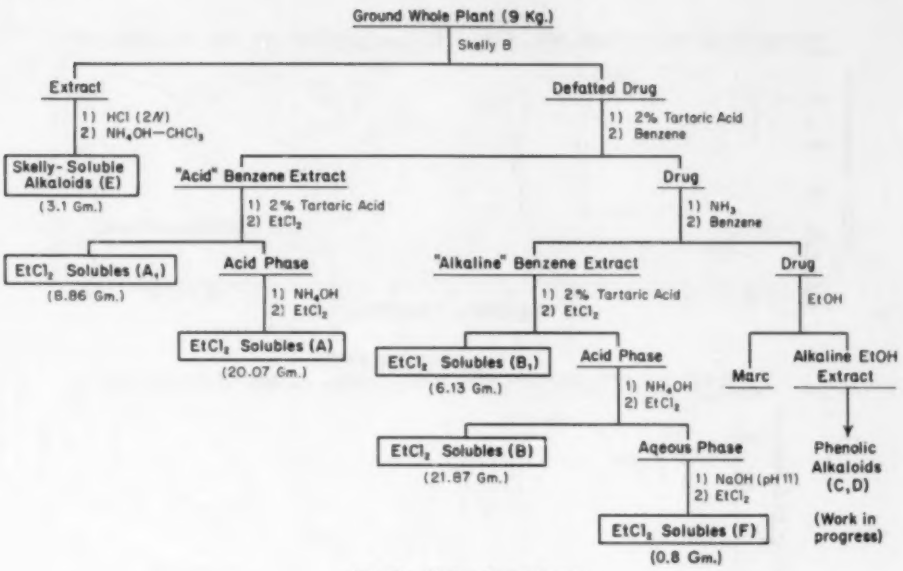


Fig. 2.—Extraction scheme.

TABLE I.—ALKALOIDS FROM *Vinca rosea* LINN.

| No. | Name | Formula |
|---|---|---|
| 1 | Ajmalicine | $C_{21}H_{24}O_3N_2$ |
| 2 | Tetrahydroalstonine | $C_{21}H_{24}O_3N_2$ |
| 3 | Serpentine | $C_{21}H_{22}O_3N_2$ |
| 4 | Lochnerine | $C_{20}H_{24}O_2N_2$ |
| 5 | Reserpine[a] | $C_{33}H_{40}O_9N_2$ |
| 6 | Akuammine[a] | $C_{22}H_{28}O_4N_2$ |

[a] Reserpine and akuammine (vincamajoridine) have not been obtained during this work.

**Alkaloidal Constituents of Vinca rosea Linn.**—The earliest chemical investigation of the plant was carried out by M. Greshoff (15), who indicated the presence of alkaloidal constituents but was unable to obtain homogenous, crystalline compounds. An advance in the phytochemistry of this plant was made by Cowley and Bennett (11) in 1928 when they were able to prepare two crystalline sulfates and a tartrate from the total alkaloidal fraction; however, no chemical or physical properties of these salts were described. In 1953, R. Paris and Moyse-Migmon reported an unidentified crystalline alkaloid from this plant (16). More recently several groups have obtained the following alkaloids (Table I): ajmalicine (17), akuammine[5] (17), tetrahydroalstonine (18), serpentine (17, 18), lochnerine (19), and reserpine[5] (20).

While our investigation was in progress, Kamat and co-workers (21) have reported on two amorphous and two crystalline alkaloids. Only one of these was described in detail and named vindoline. At about the same time Noble, Beer, and Cutts have described the alkaloid vincaleukoblastine (22) and one of us (G. H. S.) obtained three additional alkaloids, leurosine, perivine, and virosine (23). Most recently we have given details of the characterization of additional new *Vinca* alkaloids, *viz.*, catharanthine, vindolinine,[6] and lochnericine[7] and confirmed the presence of the compounds reported earlier (25) (Table II).

The alkaloids reported prior to 1958 (Table I) are well documented and do not merit further discussion. The remaining alkaloids are listed in Table II. The empirical formulas, m. p.'s, pKa's, optical rotations, and ultraviolet absorption maxima are also given. As an additional aid to the characterization of these alkaloids, their respective infrared spectra are reproduced separately (Fig. 3). The characterization of alkaloids 1–6 has been described in detail by us previously (25, 26). Perivine and virosine have been reported by one of us (G. H. S.) but no empirical formulas have been assigned (23). Perivine is now shown to be a $C_{23}H_{24}O_3N_2$ compound. This formulation was corroborated by the analysis of the hydrochloride as well as the determination of the molecular weight of this salt from X-ray data measurements of the unit cell. The spectral data of perivine indicate that this compound is another representative of 2-acyl indole alkaloids (27). Virosine has been tentatively formulated as a $C_{22}H_{26}O_4N_2$ compound. Its spectral characteristics are as yet uninterpreted and small amounts of the alkaloid did not allow any degradative studies.

**Phytochemical Analysis of the Alkaloids of Vinca rosea Linn.**—Figure 2 represents a brief outline of the scheme used in the preparation of the alkaloids. The process consists essentially of separating the plant alkaloids into those whose tartrates are soluble in organic solvents and those which are insoluble under these conditions. In each fraction ($A_1$, A, $B_1$,

[5] These alkaloids were not encountered during our investigation.

[6] The occurrence of this alkaloid has been privately communicated to us by Professor Janot, University of Paris and the name was chosen by mutual agreement with his group.

[7] An alkaloid, independently named lochnericine, has been reported by C. P. N. Nair and P. P. Pillay (24). This material appears to be identical with our lochnericine.

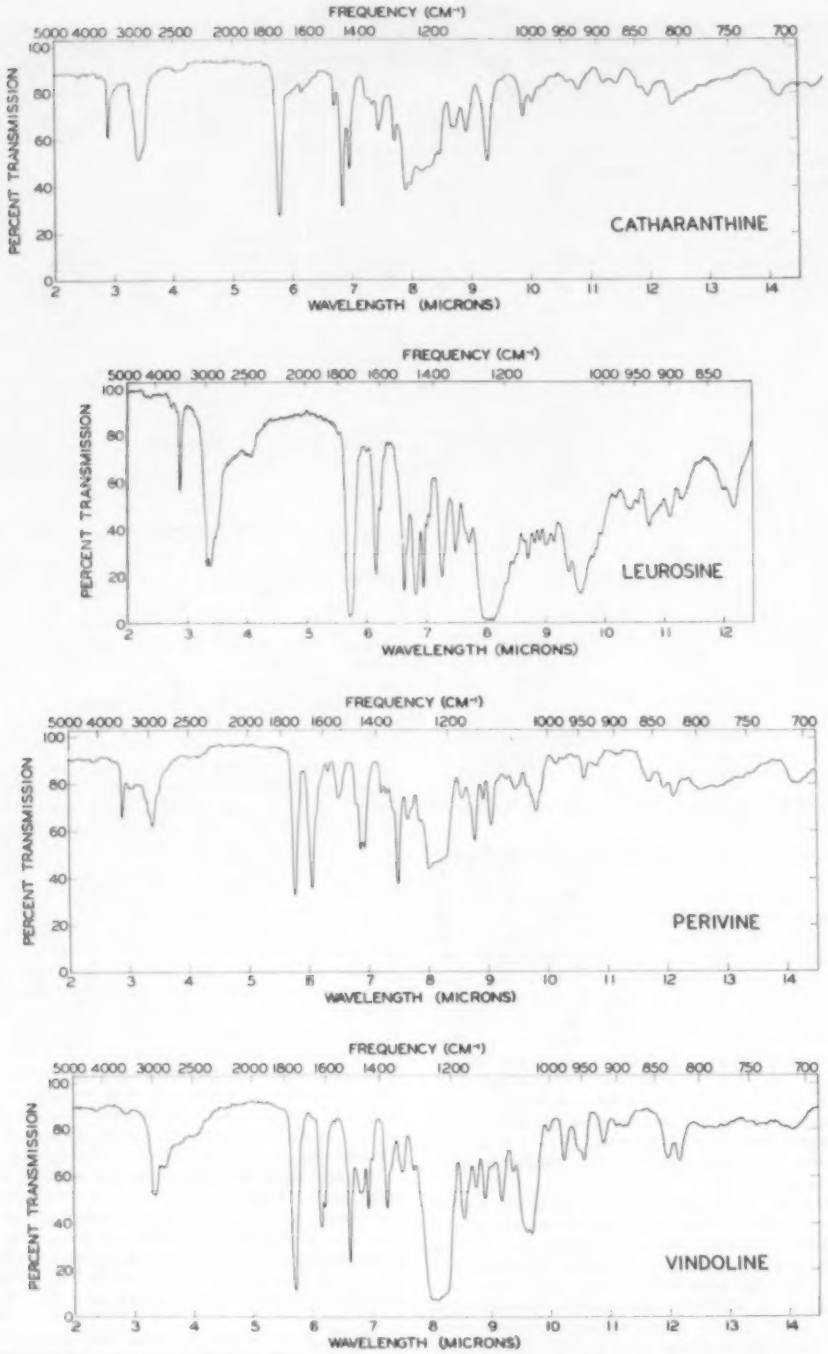


Fig. 3.—Infrared spectra of alkaloids. The infrared spectrum of vindolinine dihydrochloride was obtained in a mineral oil mull. All other spectra were recorded in chloroform in a 0.1 mm. path, using the Perkin-Elmer Model 21 spectrophotometer.

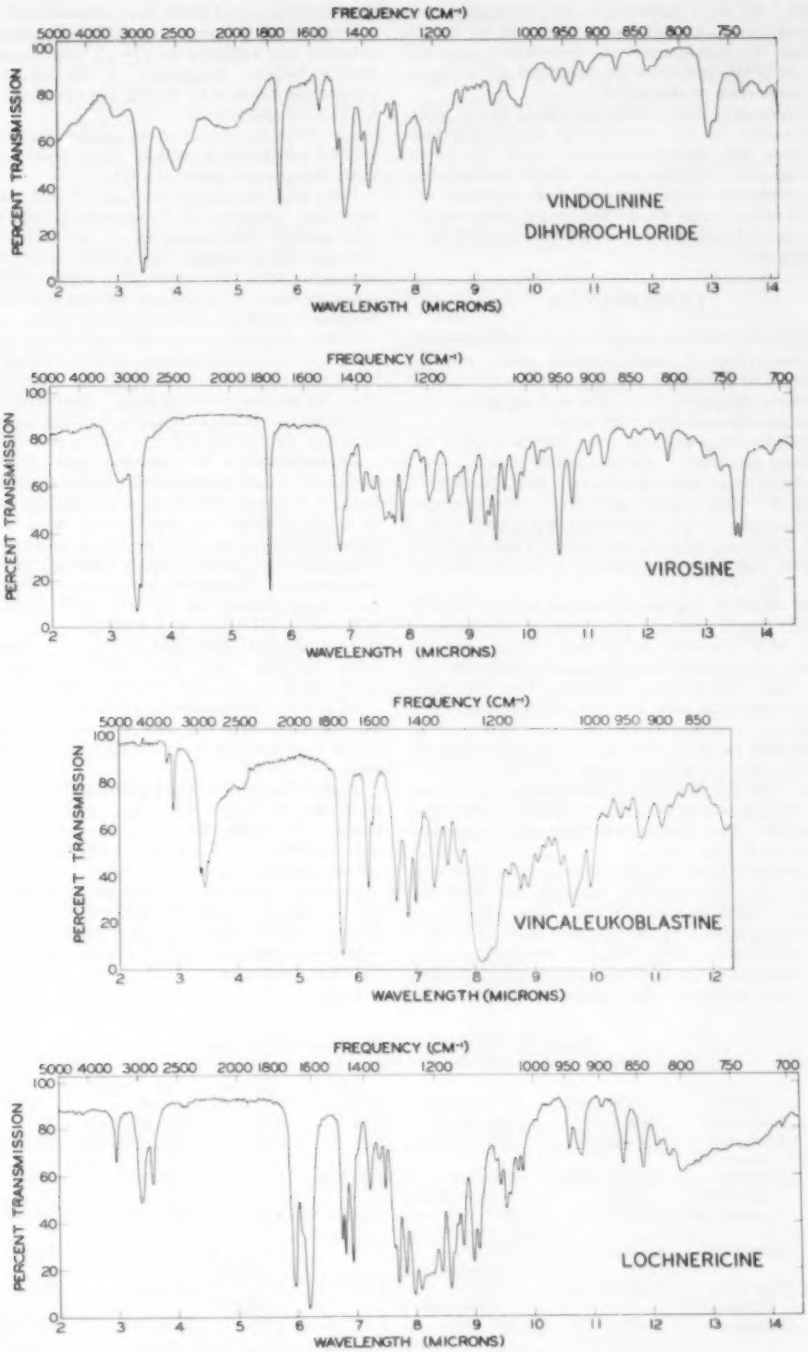
FREQUENCY (CM⁻¹)
5000 4000 3000 2500 2000 1800 1600 1400 1200 1000 950 900 850 800 750
PERCENT TRANSMISSION
VINDOLININE
DIHYDROCHLORIDE
WAVELENGTH (MICRONS)
FREQUENCY (CM⁻¹)
5000 4000 3000 2500 2000 1800 1600 1400 1200 1000 950 900 850 800 750 700
PERCENT TRANSMISSION
VIROSINE
WAVELENGTH (MICRONS)
FREQUENCY (CM⁻¹)
5000 4000 3000 2500 2000 1800 1600 1400 1200 1000 950 900 850
PERCENT TRANSMISSION
VINCALEUKOBLASTINE
WAVELENGTH (MICRONS)
FREQUENCY (CM⁻¹)
5000 4000 3000 2500 2000 1800 1600 1400 1200 1000 950 900 850 800 750 700
PERCENT TRANSMISSION
LOCHNERICINE
WAVELENGTH (MICRONS)

Fig. 3.—Infrared spectra of alkaloids. The infrared spectrum of vindolinine dihydrochloride was obtained in a mineral oil mull. All other spectra were recorded in chloroform in a 0.1 mm. path, using the Perkin-Elmer Model 21 spectrophotometer

B, etc.) the final purification and eventual separation of biological activity is achieved by careful elution chromatography on deactivated alumina. Details of this procedure are described in the experimental portion of this paper.

Preliminary observations pertaining to the interrelationship of the biologically active alkaloids leurosine and vincaleukoblastine with the indole alkaloid catharanthine and the dihydroindole alkaloid vindoline have been reported elsewhere (1). At the present time we are continuing the investigations toward a complete structure elucidation of these substances.

## EXPERIMENTAL

All known alkaloids isolated above were identified by comparison of mixed melting point, infrared spectra, and X-ray diffraction patterns with those of authentic specimens (28). The melting points were determined on a Kofler microstage.

The alkaloidal extracts were obtained using the following procedure. Ground whole plant (9 Kg.) was defatted by stirring with two 45-L. portions of Skelly B. This extract gave a (+) Mayer's test. Concentration to 3 L. and extraction with 2 *N* HCl (4 × 200 cc.) gave, after treatment of the acid with $NH_4OH$ and ether extraction, 3.1 Gm. of alkaloids (E).

The defatted drug was intimately mixed with 6 L. of 2% tartaric acid and extracted by stirring with three 48-L. portions of benzene. The combined extracts were concentrated *in vacuo* to approximately 9 L. The alkaloids were extracted from this benzene concentrate with 2% tartaric acid (two 12-L. portions). The acid extract was then extracted at pH 3.2 with two 6-L. portions of ethylene dichloride. Drying and evaporation yielded 8.86 Gm. ($A_1$).

The acid solution was made alkaline to litmus with $NH_4OH$ and was extracted with 6 L. of ethylene dichloride. The solution was dried and evaporated to give 20.07 Gm. (A).

After the "acid" benzene extract had been collected, $NH_3$ was bubbled through the drug (in benzene) until it was alkaline. The alkalinized drug was extracted by stirring with three 48-L. portions of benzene. The "alkaline" benzene extract was processed in the same manner as was that from the "acid" benzene. The yields of ($B_1$) and (B) were 6.13 Gm. and 21.87 Gm., respectively. After removal of the B fraction, the pH of the ammoniacal solution was adjusted to pH 11 with sodium hydroxide pellets. Extraction of this solution with chloroform followed by drying and evaporation gave 0.8 Gm. of alkaloid (F).

The alcoholic extract of the ammoniacal marc has yielded a number of phenolic bases which are presently being investigated (C, D).

Benzene solutions of 10 Gm. of each of these materials, obtained as amorphous powders, were subsequently chromatographed on 400 Gm. of alumina (Alcoa alumina, Grade F-20) deactivated by treatment with 12.5 cc. of 10% acetic acid in the usual manner. The eluting solvents were benzene, benzene-chloroform mixtures, chloroform, and finally chloroform-methanol mixtures. Fractions were collected, evaporated *in vacuo*, and combined, where indicated, by infrared spectroscopy. The purified alkaloids were crystallized from a suitable solvent or converted to an appropriate salt. The results are shown in Tables III, IV, V, VI, VII, and VIII.

**Characterization of Perivine and Virosine.**—*Perivine.*—From the chromatography of 20 Gm. of fraction B (Table VI) there were obtained 250 mg. of crude perivine by the elution with benzene-chloroform mixtures (1:1) (fraction 20–21). Crystallization from acetone afforded 140 mg. of crystalline perivine Two recrystallizations from methanol gave long prisms, m. p. 218–221° (decompn.), $[\alpha]_D^{25} = -121.4°$ (C = 1, $CHCl_3$).

*Anal.*—Calcd. for $C_{20}H_{22}O_3N_2$: C, 70.98; H, 6.55; N, 8.28; mol. wt.; 338.39. Found: C, 70.94; H, 6.77; N, 7.93; mol. wt.; 355 ± 10.

pK'a 7.5; electrometric titration, 66% DMF. The hydrochloride salt was prepared in a conventional manner and recrystallized from methanol-ether.

*Anal.*—Calcd. for $C_{20}H_{22}O_3N_2 \cdot HCl$: C, 63.74; H, 6.69; N, 7.43; Cl, 9.41; mol. wt. 374.86. Found: C, 63.89; H, 6.71; N, 7.51; Cl, 9.33; mol. wt. 373.5 ± 1% (X-ray). $\lambda_{max.}^{EtOH}$ 226 mμ ($E$ = 19,100) and 314 mμ ($E$ = 19,200).

*Virosine*—A careful chromatography of 50 Gm. of the fraction A (Table IV) yielded 55 mg. of pure virosine which, after two recrystallizations from chloroform-methanol, afforded long prisms, m. p. 258–261° (decompn.), $[\alpha]_D^{25} = -160.5°$ (C = 1, $CHCl_3$).

TABLE II.—ALKALOIDS FROM *Vinca rosea* LINN.

| No. | Name | Formula | M. p., °C. | $[\alpha]_D^{25}$ $CHCl_3$ | pK'a in 66% DMF | U. V. $\lambda_{max.}^{EtOH}$ in mμ | Lit. Ref. |
|---|---|---|---|---|---|---|---|
| 1 | Vindoline | $C_{25}H_{32}O_6N_2$ | 154–155 | +42 | 5.5 | 212, 250, 304 | 25 |
| 2 | Catharanthine | $C_{21}H_{24}O_2N_2$ | 126–128 | +29.8 | 6.8 | 226, 284, 292 | 25 |
| 3 | Vindolinine.2HCl | $C_{21}H_{24-6}O_2N_2 \cdot 2HCl$ | 210–212 (decompn.) | −8 ($H_2O$) | 3.3, 7.1 | 245, 300 | 25 |
| 4 | Lochnericine | $C_{21}H_{24}O_2N_2$ | 190–193 (decompn.) | −432 | 4.2 | 226, 297, 327 | 24, 25 |
| 5 | Leurosine | $C_{46}H_{58}O_9N_4$ | 202–205 (decompn.) | +72 | 5.5, 7.5 ($H_2O$) | 214, 259 | 23, 26 |
| 6 | Vincaleukoblastine | $C_{46}H_{58}O_9N_4$ | 211–216 (decompn.) | +42[a] | 5.4, 7.4 ($H_2O$) | 214, 259 | 22, 26 |
| 7 | Perivine | $C_{20}H_{24}O_3N_2$ | 180–181 | −121.4 | 7.5 | 226, 314 | 23 and this paper |
| 8 | Virosine | $C_{22}H_{28}O_4N_2$ | 258–264 (decompn.) | −160.5 | 5.85 | 226, 270 | 23 and this paper |

[a] Determined on vincaleukoblastine etherate [$C_{46}H_{58}O_9N_4 \cdot (C_2H_5)_2O$].

TABLE III.—CHROMATOGRAPHY OF ($A_1$)

| Fraction, 500 cc. ea. | Eluting Solvent | Compound | Wt., Gm. | Crystallizing Solvent |
|---|---|---|---|---|
| 1–2 | Benzene | Amorphous residues | ... | ... |
| 3–4 | Benzene | Tetrahydroalstonine | 0.112 | Methanol |
| 5–8 | Benzene | Amorphous residues | ... | ... |
| 9–14 | Benzene-chloroform (3:1) | Vindoline | 2.5 | Ether |
| 14–on | Chloroform | Amorphous residues | ... | ... |

TABLE IV.—CHROMATOGRAPHY OF (A)

| Fraction, 500 cc. ea. | Eluting Solvent | Compound | Wt., Gm. | Crystallizing Solvent |
|---|---|---|---|---|
| 1 | Benzene | Catharanthine | 0.250 | Methanol |
| 2 | Benzene | Vindolinine (as dihydrochloride) | 0.210 | Methanol-ether |
| 3–19 | Benzene | $A_1$malicine | 0.798 | Methanol |
| 20–21 | Benzene | Vindoline | 0.820 | Ether |
| 34–42 | Benzene-chloroform (1:1) | Leurosine | 0.234 | Methanol |
| 43–45 | Benzene-chloroform (1:1) | Vincaleukoblastine (as sulfate) | 0.126 | Ethanol |
| 46 | Chloroform | Virosine | 0.010 | Acetone |
| 47–52 | Chloroform-methanol | Amorphous residues | ... | ... |

TABLE V.—CHROMATOGRAPHY OF ($B_1$)

| Fraction, 500 cc. ea. | Eluting Solvent | Compound | Wt., Gm. | Crystallizing Solvent |
|---|---|---|---|---|
| 1–2 | Benzene | Amorphous residues | ... | ... |
| 3 | Benzene-chloroform (3:1) | Ajmalicine[a] | 0.059 | Acetone |
| 4–11 | Benzene-chloroform (1:1) | Amorphous residues | ... | ... |
| 12–13 | Benzene-chloroform (1:1) | β-Sitosterol | 0.008 | Methanol |
| 14–on | | Amorphous residues | ... | ... |

[a] Isolated as a monohydrate.

TABLE VI.—CHROMATOGRAPHY OF (B)

| Fraction, 650 cc. ea. | Eluting Solvent | Compound | Wt., Gm. | Crystallizing Solvent |
|---|---|---|---|---|
| 1–11 | Benzene | Oily residues | ... | ... |
| 12–13 | Benzene-chloroform (3:1) | Ajmalicine | 0.055 | Methanol |
| 14–19 | Benzene-chloroform (3:1) | Amorphous residues | ... | ... |
| 20–21 | Benzene-chloroform (1:1) | Perivine | 0.084 | Acetone |
| 22–24 | Benzene-chloroform (1:1) | Amorphous residues | ... | ... |
| 25–27 | Benzene-chloroform (1:1) | Lochnerine | 0.581 | Acetone |
| 28–38 | Chloroform | Amorphous residues | ... | ... |
| 39 | Methanol | Amorphous residues | ... | ... |

TABLE VII.—CHROMATOGRAPHY OF (E), 3 GM.

| Fraction, 150 cc. ea. | Eluting Solvent | Compound | Wt., Gm. | Crystallizing Solvent |
|---|---|---|---|---|
| 1–3 | Benzene | Yellow oil | ... | ... |
| 4–6 | Benzene | Lochnericine | 0.23 | Methanol |
| 7–18 | Benzene | Oils | ... | ... |
| 19–28 | Benzene | Tetrahydroalstonine | 0.08 | Methanol |
| 29–53 | Benzene-chloroform (3:1) | Oil | ... | ... |
| 54–58 | Benzene-chloroform (3:1) | Vindoline | 0.43 | Ether |

TABLE VIII.—CHROMATOGRAPHY OF (F), 0.8 GM.

| Fraction, 100 cc. ea. | Eluting Solvent | Compound | Wt., Gm. | Crystallizing Solvent |
|---|---|---|---|---|
| 1–10 | Benzene-chloroform (1:1) | Amorphous residues | ... | ... |
| 11 | Chloroform | Serpentine (as nitrate) | 0.02 | Water-ethanol |
| 12 | Chloroform-methanol | Amorphous residues | ... | ... |

*Anal.*—Calcd. for $C_{22}H_{26}O_4N_2$: C, 69.09; H, 6.85; N, 7.33; O, 16.73; mol. wt., 382.44. Found: C, 68.85; 69.22; H, 6.64; 6.97; N, 7.23; O, 16.99; mol. wt. 349 ± 20.

pK'a 5.85, electrometric titration, 66% DMF. $\lambda_{max.}^{EtOH}$ 226 mμ ($E$ = 29,400) and 270 mμ ($E$ = 8,500).

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# The Biological Action of Cellular Depressants and Stimulants II*

## The Kinetics of the Action of Urethane on the Growth of *Tetrahymena pyriformis*

By JOHN J. EILER, JOSEPH Z. KREZANOSKI†, and KWAN-HUA LEE

**A kinetic analysis of the action of urethane on growth shows that the rate of cell division of *Tetrahymena pyriformis*, both in the presence and in the absence of drug, is best described by a second-order rate law. The concentration of some rate-limiting factor critical to growth is lowered both by urethane and oxygen want.**

IN THE FIRST paper in this series (1), it was shown that increasing concentrations of urethane cause a progressive increase in the mean generation time of normal cells of *Tetrahymena pyriformis*. Cells which have been subcultured frequently in the presence of the drug prior to the test to produce trained cells, respond to the drug in a similar fashion, but with a decrease in sensitivity. In the tests on both types of cells (normal and trained), the quantitative estimation of the effect of the several concentrations of drug was based on the rate of cell division only during the period of logarithmic growth. Since logarithmic growth accounted for no more than 10 per cent of the total increase in population, it seemed proper to extend the examination of the data to seek both a more encompassing measure of drug action and, if possible, additional information concerning the mechanism of action. Such an examination is presented here.

* Received May 18, 1959, from the School of Pharmacy, University of California Medical Center, San Francisco 22.
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### DATA, CONCEPTS, AND DISCUSSION

The growth data which form the basis of this paper have been taken from our previous report (1). The cells were grown in 2,000-ml. Provitsky flasks containing either 750 ml. of a proteose-peptone-yeast extract medium or 750 ml. of the medium to which had been added known amounts of urethane. The flasks were inoculated with *Tetrahymena pyriformis* either from cultures of normal cells or from cultures of trained cells so as to yield, in either case, an initial population of 1,000 cells/ml. The cultures were stored in the dark at 27° without agitation except for the periodic withdrawal of samples for the purpose of estimating the cell population.

The increases in cell count in the absence and in the presence of the several concentrations of urethane are presented in Fig. 1, where the ratio of the time-dependent population ($Pt$) to the initial population ($Po$) is plotted on a logarithmic scale *versus* time. The slope of the linear portion of each of the curves constitutes a quantitative measure of the effect of a given concentration of

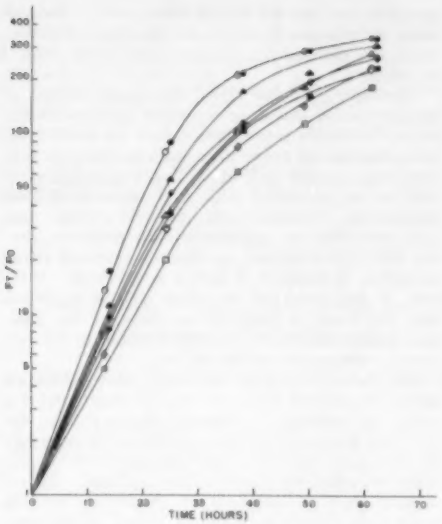


Fig. 1.—The effect of several concentrations of urethane on trained and normal cells. The time-dependent cell count ($P_t$) divided by the initial cell count ($P_o$) is plotted on a logarithmic scale. Normal cells, ○ = control; Δ = 0.07 *M*; ◇ = 0.08 *M*; and □ = 0.09 *M*. Trained cells, ● = control; ▲ = 0.07 *M*; ◆ = 0.08 *M*; and ■ = 0.09 *M*.

drug. The slopes are reported as first-order reproduction constants ($k_1$) in Table I.

It is to be noted in each of the curves of Fig. 1 that adherence to first-order kinetics is limited to about the first five generations. A continual decrease in the reproduction constant (slope) is observed to take place after the attainment of a population of about 20,000–25,000 cells/ml. About the same limit to exponential growth seems to hold in all cases. Since the maximum populations ($Px$) attained ranged between 275,000–363,000 cells/ml. (see Table I), it is clear that nonexponential growth represents over 90% of the increase in cell population.

The downward concavity of the growth curve, when plotted in accordance with first-order kinetics, suggests the possibility that growth may conform to a rate law of some higher order. Growth curves following a second-order rate law (2, 3, 4) or even higher (3, 5) have been reported frequently. Indeed, Browning, *et al.* (6), have observed conditions under which *Tetrahymena* cells may grow according to second-, third-, or higher order kinetics.

TABLE I.—THE VALUES OF SEVERAL CONSTANTS RELATED TO THE RATE OF GROWTH OF *Tetrahymena pyriformis* AND ITS INHIBITION BY URETHANE

| Cell Type | Concn. Urethane, Moles/Liter | $k_1$ hr.$^{-1}$ | $P_x$ × 10$^3$ | $P_x/k_1$ × 10$^3$ | $k_1 \times 10^{-6}$ ml./cell/hr. |
|---|---|---|---|---|---|
| Normal | 0.0 | 0.417 | 363 | 873 | 0.37[a] |
| Normal | 0.07 | 0.329 | 313 | 950 | 0.37[a] |
| Normal | 0.08 | 0.288 | 273 | 946 | 0.37[a] |
| Normal | 0.09 | 0.259 | 254 | 978 | 0.37[a] |
| Trained | 0.0 | 0.417 | 362 | 873 | 0.37[a] |
| Trained | 0.07 | 0.356 | 326 | 915 | 0.37[a] |
| Trained | 0.08 | 0.336 | 311 | 930 | 0.37[a] |
| Trained | 0.09 | 0.309 | 275 | 890 | 0.37[a] |

[a] Calculated from slope in Fig. 2.

After the trial of several possible rate laws, success was achieved with the use of the rate law applicable to second-order autocatalytic reactions (4, 7) which, using the symbols employed by Browning, *et al.* (5), describes the time-dependent increase in cell number as follows:

$$P_t = \frac{P_x}{1 + \frac{P_x - P_0}{P_0} \cdot e^{-P_x k_2 t}} \quad \text{(Eq. 1)}$$

where $P_x$ represents the maximum population and $k_2$ the second-order reproduction constant. The other symbols are as used already.

For purposes of making calculations and plotting the results, a logarithmic form of the equation, as follows, is more useful:

$$\frac{2.3}{P_x} \log \frac{P_t}{P_x - P_t} \cdot \frac{P_x - P_0}{P_0} = k_2 t \quad \text{(Eq. 2)}$$

Table II contains the values for the first member of Eq. 2 for the data presented graphically in Fig. 1. It is to be observed that all the values related to a given time are very closely alike. Presumably all populations of cells, normal and trained, in the presence and in the absence of drug, multiplied according to the same law. The agreement of the data with values calculated assuming second-order kinetics may be observed in Fig. 2 where, due to the frequency of the coincidence of points (see Table II), we have plotted only the average of the points for a given time for each type of cell. The slope, or the average value for $k_2$, amounted to 0.37 × 10$^6$ ml./cell/hour.

While some concern may be expressed over the fact that the curve does not meet the origin, a fact which may be due to the lack of control of the pH of the cultures (8) and other factors, there can be little doubt concerning the utility of Eq. 2

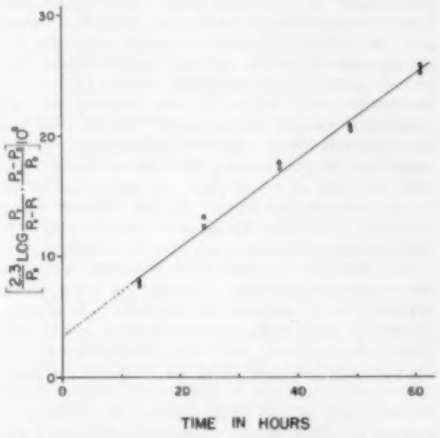


Fig. 2.—The results given in Fig. 1 are plotted according to Eq. 2, with the first member of the equation as ordinate and the time in hours as the abscissa.

TABLE II.—THE VALUES FOR $k_2 I$ CALCULATED BY USE OF THE FIRST MEMBER OF EQ. 2 FROM THE DATA PRESENTED IN FIG. 1

| Cell Type | Concn. Urethane | $k_2 I \times 10^2$ 13 hr. | 24 hr. | 37 hr. | 49 hr. | 61 hr. |
|---|---|---|---|---|---|---|
| Normal | 0.0 | 7.4 | 12.8 | 17.3 | 20.2 | 24.9 |
| Normal | 0.07 | 6.7 | 11.7 | 16.3 | 19.3 | 25.2 |
| Normal | 0.08 | 6.8 | 12.9 | 17.7 | 21.1 | 25.7 |
| Normal | 0.09 | 6.3 | 11.9 | 17.1 | 20.8 | 25.2 |
| Trained | 0.0 | 9.0 | 13.3 | 17.5 | 20.4 | 24.4 |
| Trained | 0.07 | 7.5 | 13.3 | 18.1 | 20.4 | 27.3 |
| Trained | 0.08 | 7.4 | 13.0 | 16.8 | 20.2 | 24.5 |
| Trained | 0.09 | 7.9 | 13.6 | 18.7 | 21.9 | 27.0 |

in describing the growth of the cells, both in the presence and in the absence of drug.

In considering the possible biological significance (2, 3, 4) of second-order kinetics, it may be assumed that the rate of cell multiplication is proportional both to the cell population and to some limiting factor. According to Browning, *et al.* (5), the necessary relation can be set forth as follows:

$$\frac{dP_t}{d_t} = k_2 P_t F_t \qquad \text{(Eq. 3)}$$

where $F_t$ is the concentration of the limiter.[1]

While no attempt will be made to consider more precisely the nature of $F_t$ at this time, there is little doubt that this factor is related to the rate of oxygen consumption, which in our studies has been limited either by the supply of oxygen (9, 10, 11) or by the effect of the drug (1). With the aid of even such a sketchy knowledge of $F_t$, it becomes possible to explain several aspects of the data presented in Fig. 1.

Consider first the curve for the rates of division for both the normal and the trained cells in the absence of drug (Fig. 1). During the first four generations of growth, the rate of solution of oxygen, which is limited by the surface of the culture medium exposed to the atmosphere, exceeds the rate of consumption of oxygen, which in turn is determined by the concurrent cell population. Under these conditions $k_2 F_t = k_1$ (drug-free value) and since the medium is saturated with oxygen, the magnitude of $F_t$ is maximal and constant, and growth proceeds exponentially (first order) at a maximum rate. When, in our studies, the cell population exceeds about 20,000–25,000 cells/ml. (between the fourth and fifth generation), the demand for oxygen exceeds its rate of supply and both the rate of respiration and the value of its closely related parameter $F_t$ fall. Under these conditions, second-order growth sets in and continues until the maximum population is reached.

The situation is very little different with the drug-depressed growths. During the early phase of growth in the presence of drug, $k_2 F_t = k_1$ (characteristic for each drug concentration) as in the case of drug-free growth, but with the difference that although constant, the value for $F_t$ is not maximal. Indeed, the value for $F_t$ is inversely related to the concentration of the drug due to the respiratory depressing effect of the drug (1). The drug-dependent values of $F_t$ give rise to the values of $k_1$ characteristic for each drug concentration. Second-order growth sets in, as in the drug-free situation, when the demand for oxygen exceeds the rate of its supply.

According to these views, the linear portion of the curves for the drug-depressed growths should extend to higher population values the greater the concentration of drug, since each increase in concentration would permit a larger population of cells to be associated with a given level of total respiration. Unfortunately, our cell count data were not taken at sufficiently close intervals, during the critical period, to furnish evidence either against or in support of such a proposition. However, it was observed, as might well be expected, that the linear portion of the curve in the drug-free system was extended with increase in the surface to volume ratio of the culture.

The indication that the same growth-limiting factor is affected both by oxygen want and the action of urethane is strongly supported by the fact that growth under both conditions is described by the same equation and characterized by the same second-order reproduction constant. In accepting such a statement, it should be kept in mind that the primary site of action of the drug need not be on electron transport. A primary inhibition of some growth-essential endergonic process would have the same effect.

Although exponential growth accounted for less than 10% of the total increase in cell population, the first-order growth constant appears to have furnished a valid index of the quantitative effects of the drug. As is shown in Table I, the maximum populations attained ($P_x$) decreased progressively as the concentration of drug increased. The variation in $P_x$ for both types of cells is similar to the variation in $k_1$. Indeed, the listed values for $P_x/k_1$ in Table I are nearly constants. The constancy of this relationship indicates that $P_x$ and $k_1$ are equally good measures of the quantitative effect of urethane. Poole and Hinshelwood (12) observed a similar parallel variation in the mean generation time ($0.693/k_1$) and the maximum population caused by growth-depressing agents. The explanation offered by them may well hold for the action of urethane.

One further point might be made; although the data are limited, the plots for either $k_1$ or $P_x$ *versus* concentration are linear. This is in conformity with what is frequently observed for narcotic drugs (13).

## SUMMARY

1. A kinetic analysis of the effect of urethane on the rate of cell division of *Tetrahymena pyriformis* shows that both the drug-free and the drug-inhibited growth are best described by a second-order autocatalytic rate law.

2. The idea is advanced that both urethane and oxygen-want influence the concentration of a rate-limiting factor necessary for growth.

3. Both the first-order reproduction constant ($k_1$) and the magnitude of the maximum population ($P_x$) are valid measures of the quantitative effects of urethane.

[1] In the derivation of Eq. 2, $F_t$ is equated to $P_x - P_t$. In a sense, it is a measure of the unborn population since at $t = \infty$, $F_t = 0$ and $P_t = P_x$.

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# A Note on the Synthesis and Pharmacological Activities of a Vinylog of Nikethamide and Some Related Substances*

By HUGH BURFORD†, ALTA R. GAULT, S. T. COKER‡, and W. LEWIS NOBLES

THE RELATIONSHIP of physical and chemical properties of a compound and its vinylogs is well known (1); interest has been manifest by others (2) in attempting to relate the pharmacological actions of a chemical substance and its vinylogs. Previously, we (3) have reported on such studies in the areas of antitubercular agents, analgesics, antispasmodics, general antibacterial agents, and thyroxine analogs.

The present study was designed in an effort to extend our previous work with vinylogous compounds to vinylogs of nicotinic acid and its diethylamide.

With respect to the pharmacological action of pyridyl-3-acrylic acid, a vinylog of nicotinic acid, both *in vitro* antibacterial screening and an anti-inflammatory test using the granuloma pouch method were conducted with this compound. No activity was demonstrated in either test; the $LD_{50}$ of this substance intraperitoneally in mice was determined to be 1,900 mg./Kg.[1]

In a preliminary study in our laboratory, the effect of this vinylog of nikethamide was determined by intravenous injection into rats and rabbits. With the rats, a closed respiratory system was used; on rabbits, an open system was utilized. In both cases, nikethamide was used as the control standard. The vinylog was found to mimic the action of nikethamide on a qualitative scale; the respiratory rate was increased in both types of animals to approximately the same degree with equivalent doses of each drug.

The results of additional tests with this vinylog are summarized as follows[1]: (*a*) CNS: the compound appears to have slight sedative and muscle relaxant properties; no significant behavorial changes in cats with an oral dose of 32 mg./Kg. were noted. (*b*) Pharmacodynamic: the profile of this compound in this test indicated it to be a weak ganglionic blocking agent. (*c*) Antiserotonin: in this test, the compound has very little activity.

## EXPERIMENTAL

**Pyridyl-3-acrylic Acid Diethylamide Hydrochloride.**—Pyridyl-3-acrylic acid was prepared in 74% yield according to the method of Panizzon (4). This acid was converted to the corresponding acid chloride by heating it at reflux temperature with excess thionyl caloride. The crude acid chloride was allowed to react with diethylamine on a 1:2 molar basis by placing 10 Gm. (0.06 mole) of the acid chloride in 250 ml. of dry benzene in a three-neck flask equipped with a rapid stirrer, reflux condenser, and dropping funnel. Seven and six-tenths grams (0.12 mole) of diethylamine in dry benzene was added dropwise to the mixture. After the addition of diethylamine, the contents of the flask were brought to reflux for fifteen minutes and the solution filtered while hot to remove the diethylamine hydrochloride. The filtrate was shaken with a 30% solution of sodium hydroxide. The benzene layer was removed and dried over anhydrous sodium carbonate. Benzene was removed from the extract and the residue distilled. The product distilled in the range of 155–158° at 0.2 mm. (Panizzon (4) reports 145° at 0.1 mm.). Anhydrous hydrogen chloride was bubbled through a solution of this material dissolved in dry benzene to precipitate the hydrochloride of the desired compound. Approximately 12 Gm. (62%) of the product, m. p. 228–230°, was obtained.

*Anal.*—Calcd. for $C_{12}H_{16}N_2O \cdot HCl$: C, 59.87; H, 7.12. Found: C, 59.53; H, 7.31.

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Abstracted, in part, from a thesis submitted to the Graduate School of the University of Mississippi by Hugh Burford in August, 1956, in partial fulfillment of the requirements for the M.S. degree.


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[1] Pharmacological results are made possible through the courtesy of Dr. George Naimark, White Laboratories, Inc. and Dr. R. M. Taylor, Schering Corp.

# A Note on the Occurrence of Gramine in *Acer rubrum* L.*

BY IRWIN J. PACHTER

In a previous communication (1) it was reported that the alkaloid gramine occurs in the leaves of the silver maple tree, *Acer saccharinum* L. Since alkaloids had not been reported previously in plants of the maple family, it was of interest to us to check other maples for alkaloid content.

Tests on the leaves and stems of *Acer rubrum* L.[1] showed the presence of traces of alkaloids. The winged fruit[1] gave a stronger test and was therefore subjected to analysis.

From 120 Gm. of dried ground fruit there was isolated 37 mg. of a crystalline alkaloid, m. p. 130–131°. The alkaloid was found to be identical with a synthetic specimen of gramine through mixed melting point determination and comparisons of infrared and ultraviolet spectra. The picrate derivatives of the natural and synthetic specimens were prepared and found to be identical.

## EXPERIMENTAL

A 120-Gm. sample of dried fruit of *Acer rubrum* L. was finely ground in a Waring Blendor and allowed to stand under hexane for one day. The hexane solution was removed by filtration. It gave a negative test for alkaloids and was discarded. The marc was then stirred under reflux with 95% ethanol for three hours and filtered. Extraction with ethanol was repeated five times. The alcoholic solutions were combined and evaporated to dryness. The residue was dissolved by shaking with 200 ml. of ethyl acetate and 200 ml. of 2% aqueous ammonia. Twenty grams of sodium chloride was added to hasten separation of the organic and aqueous phases. The ethyl acetate layer was shaken with three 100-ml. portions of 2% hydrochloric acid. The acid solutions were combined, made strongly basic with concentrated ammonia, and extracted with three 100-ml. portions of ether. The ethereal solutions were combined, dried over magnesium sulfate, and evaporated to dryness. The residue crystallized. It was recrystallized from benzene to yield 37 mg. of crystalline gramine, m. p. 130–131°. Upon admixture with an authentic sample of gramine, m. p. 131–132°, there was no depression of melting point.

The alkaloid formed a picrate, m. p. 140–141°, which did not depress the melting point of authentic gramine picrate.

* Received September 4, 1959 from the Research and Development Division, Smith Kline and French Laboratories, Philadelphia 1, Pa.

The author is grateful to Mr. Herman Farber for laboratory assistance.

[1] The samaras were collected in February and the leaves and stems in April in the Tallahassee, Florida, area by Professor R. K. Godfrey of the Florida State University. Herbarium specimens, Nos. 58,200 and 58,323, have been deposited at the herbarium of the Florida State University.

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# Book Notices

*Symposium on Nuclear Sex.* Edited by D. Robertson Smith and William M. Davidson. Interscience Publishers, Inc., 250 Fifth Ave., New York 1, N. Y., 1958 xvii + 188 pp. 14 x 21.5 cm. Price $3.50.

The papers and discussions at the symposium are introduced with a reference to the classical paper of Polani, Hunter, and Lennox which showed that some women with what was then called ovarian agenesis seemed to be genetic males. The papers are grouped under the headings: Cytological and genetic aspects of nuclear sex, Application to the study of intersex and related states, and Application to the study of tumors.

*Clinical Evaluation of New Drugs.* Edited by S. O. Waife and Alvin P. Shapiro. Paul B. Hoeber, Inc., Medical Book Dept. of Harper & Brothers. 49 East 33rd St., New York 16, N. Y., 1959. x + 223 pp. 15.5 x 23.5 cm. Price $7.50.

The methods and standards by which new therapeutic agents can be evaluated are presented. Each of the 14 chapters in the book has been written by an author experienced in the area covered. The steps leading from the inception of a drug to its application are adequately discussed, and criteria are presented by which the medical practitioner can judge critically how thoroughly a new agent has been investigated. The ethics of human experimentation, placebos, evaluation of subjective responses, statistical problems, and funds for research are some of the subjects that have been covered. On clinical trials in practice, chapters are devoted to infectious and gastrointestinal diseases and to nutritional and metabolic, cardiovascular, and psychiatric disorders. This book should be of special importance to all who have an interest in new drugs.

*Mechanisms of Hypersensitivity.* Henry Ford Hospital International Symposium. Edited by Joseph H. Shaffer, Gerald A. LoGrippo, and Merrill W. Chase. Little, Brown and Co., 34 Beacon St., Boston 6, Mass., 1959. xx + 754 pp. Price $18.50.

Sixty-five specialists working in the fields of allergy, dermatology, immunology, and allied sciences have contributed to this book. Their latest findings on immunologic processes as they pertain to problems of hypersensitivity are presented. The individual topics cover and integrate a large number of subjects, such as factors that condition the individual to undergo sensitization, the diverse types of antibodies, the apparent molecular heterogeneity within the recognized types and newer methods of detecting them, new aspects of the physiological control of histamine in allergy, and evidence for a role of serotonin. Many other topics related to the mechanisms of hypersensitivity are discussed, and the book should serve adequately as a standard reference on the subject.

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